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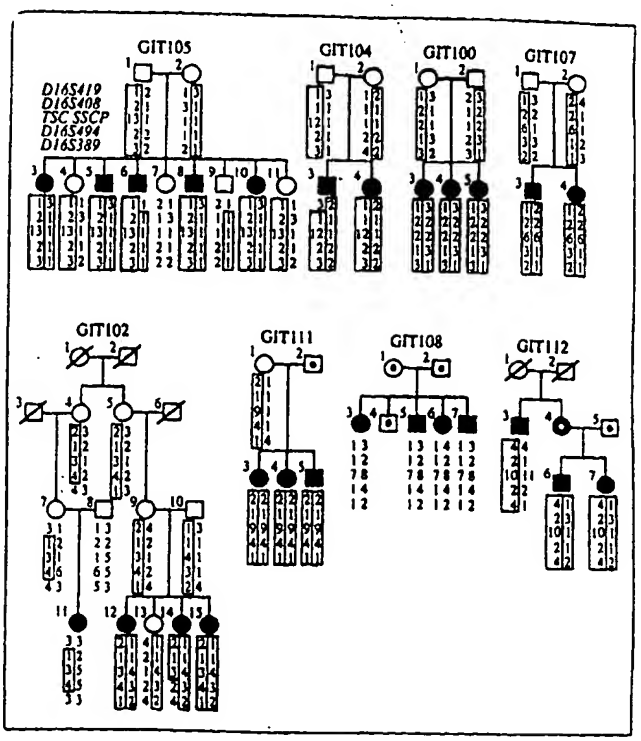
(54) Title: METHOD TO DIAGNOSE AND TREAT PATHOLOGICAL CONDITIONS RESULTING FROM DEFICIENT ION TRANSPORT

(57) Abstract

The present invention is based, in part, on the identification of the roles of the human thiazide-sensitive Na-Cl cotransporter, TSC; the human ATP-sensitive $K^+$ channel, ROMK; and the human Na-K-2Cl cotransporter, NKCC2 in causing pathological condition associated with abnormal ion transport, particularly Bartter's Syndrome, Gitelman's Syndrome, hypokalaemic alkalosis, hypokalaemic alkalosis with hypercalciuria, kidney stones, high blood pressure, osteoporosis and sensitivity to diuretic-induced hyperkalaemia. The present invention specifically provides the amino acid sequence of several human wild-type and altered variants of the TSC, NKCC2 and ROMK proteins as well as the nucleotide sequence that encodes these variants that can be used in diagnosing ion transport disorders.



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# METHOD TO DIAGNOSE AND TREAT PATHOLOGICAL CONDITIONS RESULTING FROM DEFICIENT ION TRANSPORT

## Technical Field

The invention relates to the fields of detecting and treating homozygous and heterozygous genetic deficiencies in ion transport, particularly alterations in nucleic acid molecules and proteins that give rise to various forms of Bartter's Syndrome and Gitelman's Syndrome. More specifically, the invention provides compositions and methods for determining whether an individual is affected by or carriers a mutation in one or more genes involved in ion transport.

## Background Art

Background

In higher eukaryotes the maintenance of the proper ionic composition and volume of the intravascular space is critical for normal neuromuscular function and delivery of oxygen and nutrients to tissues. The kidney plays a dominant role in determining the long-term set points of fluid and electrolyte balance, maintaining homeostasis despite wide variation in environmental exposure. Derangements in these components of kidney function are likely to underlie a number of clinical disorders ranging from altered blood pressure due to changes in intravascular volume to abnormalities in electrolyte homeostasis. Examination of mendelian disorders of fluid and electrolyte homeostasis provides the opportunity to dissect the fundamental mechanisms governing this process. This effort provides insight into basic physiology and also identify targets in which more subtle variation might commonly have effects in the population (Lifton, R.P., *Proc. Nat. Acad. Sci. U.S.A.* 92:8545-8551 (1995)).

Bartter's Syndrome is an autosomal recessive disorder featuring hypokalaemic metabolic alkalosis with salt wasting (Bartter, F.C., *et al.*, *Am. J. Med.* 33:811-828 (1962)). Affected patients have been shown to have a diverse array of additional metabolic abnormalities, including elevated plasma renin activity (Bartter, F.C., *et al.*, *Am. J. Med.* 33:811-828 (1962)), hyperaldosteronism (Goodman, A.D., *et al.*, *N. Eng. J. Med.* 281:1435-1439 (1969)), altered prostaglandin metabolism (Dunn, M.J., *Kid. Int.* 19:86-102 (1981)), elevated levels of atrial natriuretic peptide (Imai, M., *et al.*, *J. Ped.* 74:738-749 (1969)), Graham, R.M., *et al.*, *Hypertension* 8:549-551 (1986)), abnormal platelet function (Rodrigues Pereira, R., *et al.*, *Am. J. Med. Gen.* 15:79-84 (1983)), and insensitivity to the vasoconstrictive effects of angiotensin II and norepinephrine (Bartter, F.C., *et al.*, *Am. J. Med.* 33:811-828 (1962); Silverberg, A.B., *et al.*, *Am. J. Med.* 64:231-235 (1978)). Symptoms and signs of disease in affected patients reflect these diverse physiologic findings, and include signs of intravascular volume depletion (Bettinelli, A., *et al.*, *J. Pediatr.* 120:38-43 (1992)), seizures (Iwata, F., *et al.*, *Acta Paed. Japonica* 35:252-257 (1993)), tetany (Bettinelli, A., *et al.*, *J. Pediatr.* 120:38-43 (1992)), muscular weakness (Marco-Franco, J.E., *et al.*, *Clin. Neph.* 42:33-37 (1994)), paresthesias (Zarraga Larrondo, S., *et al.*, *Nephron* 62:340-344 (1992)), and joint pain with chondrocalcinosis (Smilde, TJ., *et al.*, *J. of Rheum.* 21:1515-1519 (1994)). Persistent abnormalities in electrolyte composition have resulted in stunted growth and mental retardation in some affected subjects (Simopoulos, A.P., *et al.*, *Nephron* 23:130-135 (1979)). These profound derangements in electrolyte homeostasis can lead to the misdiagnosis of bulimia and/or diuretic abuse in affected individuals (Okusa, M.D. and Bia, M.J. Bartter's Syndrome. In Hormone Resistance and Other Endocrine Paradoxes, eds. Cohen, P. and Foa, P. 231-263 (Springer Verlag, New York, 1987)).

Bartter's Syndrome has been proposed to be a heterogeneous entity with at least two subsets, Gitelman's Syndrome (Gitelman, H.J., Graham, J.B., and Welt, L.G. A new familial disorder characterized by hypokalaemia and hypomagnesemia. *Trans. Assoc. Am. Phys.* 79:221-235 (1966)) and "true Bartter's Syndrome"

(Bettinelli, A., *et al.*, *J. Pediatr.* 120:38-43 (1992)). Gitelman's Syndrome refers to the predominant subset of patients with hypokalaemic alkalosis in conjunction with hypocalciuria and hypomagnesemia, while true Bartter's Syndrome refers to patients with normal or hypercalciuria and typically normal magnesium levels. True Bartter's patients are said to present clinically at early ages (less than 5 years) with signs of vascular volume depletion, while Gitelman's Syndrome patients typically present at older ages without overt hypovolemia (Bettinelli, A., *et al.*, *J. Pediatr.* 120:38-43 (1992)). Nonetheless, the overlapping features of these disorders has resulted in considerable confusion and controversy regarding their classification, with many patients having features of Gitelman's Syndrome being labeled as having Bartter's Syndrome in the literature (Rudin, A., *et al.*, *Scand. J. Urol. Nephrol.* 22:35-39 (1988)). The pathogenesis of these disorders has remained uncertain, with wide speculation as to which observed abnormalities are primary and which are secondary consequences of underlying primary abnormalities (Clive, D.M. *Am. J. Kid. Dis.* 25:813-823 (1995)). Presently, there is not an easy method for differentiating these disorders; differentiation being based solely on evaluating the clinical symptoms that are presented.

Dissection of the physiology of renal electrolyte homeostasis has identified a number of potential candidate genes for Gitelman's Syndrome and Bartter's Syndrome; prior studies have investigated genes encoding atrial natriuretic peptide and the angiotensin II receptor (AT1) (Graham, R.M., *et al.*, *Hypertension* 8:549-551 (1986), Yoshida, H., *et al.*, *Kid. Int.* 46:1505-1509 (1994)). Another attractive candidate gene is the thiazide-sensitive Na-Cl cotransporter of the distal convoluted tubule (thiazide-sensitive cotransporter, TSC), which is believed to be the principle mediator of sodium and chloride reabsorption in this nephron segment, accounting for a significant fraction of net renal sodium reabsorption (Ellison, D.H., *Ann. Int. Med.* 114:886-894 (1991)). This cotransporter is the target of thiazide diuretics, one of the major classes of agents used in the treatment of high blood pressure. cDNAs encoding the TSC have recently been cloned from flounder bladder and rat kidney

(Gamba, G., *et al.*, *Proc. Natl. Acad. Sci. U.S.A.* 90:2749-2753 (1993); Gamba, G., *et al.*, *J. Biol. Chem.* 269:17713-17722 (1994)). The encoded protein from rat comprises 1002 amino acids, and contains twelve putative transmembrane domains, with long intracellular amino and carboxy termini. Similarities in some features of patients with Gitelman's Syndrome and patients receiving thiazide diuretics raise the possibility that mutation in *TSC* causing loss of function could result in Gitelman's Syndrome. This consideration motivates examination of *TSC* as a candidate gene for Gitelman's Syndrome.

In Example 1, it is demonstrated that Gitelman's Syndrome is a genetically homogeneous autosomal recessive trait caused by loss of function mutations in the thiazide-sensitive Na-Cl cotransporter protein (TSC) located in the renal distal convoluted tubule. The predominant clinical and physiologic abnormalities seen in these patients can be explained by the resultant salt wasting from this nephron segment.

These observations in patients with Gitelman's Syndrome leave open the question of whether Bartter's Syndrome is an allelic variant of Gitelman's Syndrome or is due to mutation in a different gene. The occurrence of salt wasting, impaired urinary concentration and calcium wasting in Bartter's patients suggests a primary renal tubular defect in the thick ascending limb (TAL) of the loop of Henle (Gill, J.R., *et al.*, *Am. J. Med.* 65:766-772 (1978)). The absorptive variant of the bumetanide-sensitive Na-K-2Cl cotransporter (NKCC2 , also known as SLC12A1) is the primary mediator of sodium and chloride reabsorption in this nephron segment (Greger, R., *Physiol. Rev.* 65:760-797 (1985)), and loss of function of this cotransporter could produce many of the features seen in affected patients. Indeed, loop diuretics, specific antagonists of this cotransporter, can produce electrolyte disturbances very similar to those seen in patients with Bartter's Syndrome (Greger, R., *et al.*, *Klin. Woschenschr.* 61:1019-1027 (1991)).

cDNA's encoding NKCC2 have recently been cloned from rat (Gamba, G. *et al.*, *J. Biol. Chem.* 26:17713-17722 (1994)), rabbit (Payne, J.A., *et al.*, *Proc. Natl.*

*Acad. Sci. (U.S.A.)* 91:4544-4548 (1994)) and mouse (Igarashi, P. *et al.*, *Am. J. Physiol.* 269:F405-F418 (1995)); a secretory variant of this cotransporter, NKCC1 (also known as SLC12A2) has also been cloned from shark (Xu, J.-C. *et al.*, *Proc. Natl. Acad. Sci. (U.S.A.)* 91:2201-2205 (1994)) and human (Payne, J.A. *et al.*, *J. Biol. Chem.* 270:17977-17985 (1995)). All members of this family have 12 putative transmembrane spanning domains and also show structural and sequence similarity to TSC. By investigation of families with Bartter's Syndrome, Example 2 demonstrates that this variant of inherited hypokalaemic alkalosis is caused by mutations in the gene encoding NKCC2. These findings explain the molecular basis of this disease and suggest possible clinical features of the more common heterozygous carrier state.

In Examples 1 and 2, evidence is presented that demonstrates that autosomal recessive hypokalaemic alkalosis with salt wasting and low blood pressure can be caused by mutations in either of two genes. Mutations in *TSC* (locus symbol SLC12A3, sometimes referred to as *NCCT*), encoding the thiazide-sensitive Na-Cl cotransporter of the renal distal convoluted tubule, cause Gitelman's Syndrome, featuring salt wasting and hypokalaemic alkalosis associated with marked hypocalciuria and hypomagnesemia (Example 1 and Simon, D.B. *et al.*, *Nature Genet.* 12:24-30 (1996)). Mutations in *NKCC2* (locus symbol SLC12A1), encoding the renal bumetanide-sensitive Na-K-2Cl cotransporter of the thick ascending limb of Henle's loop (TAL), cause Bartter's Syndrome, featuring salt wasting and hypokalaemic alkalosis associated with marked hypercalciuria and frequently nephrocalcinosis (Example 2 and Simon, D.B., *et al.*, *Nature Genet.* 13:183-188 (1996)). While Bartter's patients typically are born prematurely with polyhydramnios and show marked dehydration in the neonatal period, Gitelman's patients typically present at older ages with neuromuscular signs and symptoms (Wang, W., *et al.*, *Ann. Rev. Physiol.* 54:81-96 (1992)).

Mutations in genes whose products regulate activity of either of these cotransporters could potentially lead to similar clinical phenotypes. An apical ATP-sensitive $K^+$ channel has been implicated as one such regulator of the Na-K-2Cl

cotransporter in the TAL (Wang, W., *et al.*, *Ann. Rev. Physiol.* 54:81-96 (1992), Giebisch, G., *Kidney Int.* 48:1004-1009 (1995)). Since $K^+$ levels in the TAL are much lower than levels of $Na^+$ and $Cl^-$, availability of tubular $K^+$ is rate limiting for cotransporter activity; $K^+$ entering the cell from the tubule must be "recycled" to the lumen in order to permit sustained cotransport activity. This key role of $K^+$ channels in the regulation of cotransporter activity is demonstrated by the ability of potassium channel antagonists to virtually abolish Na-K-2Cl cotransporter activity, Giebisch, G., *Kidney Int.* 48:1004-1009 (1995)).

An inwardly rectifying $K^+$ channel (IRK) bearing many features of this regulatory channel (low single channel conductance, activation by low levels of ATP and protein kinase A (PKA), and insensitivity to voltage and calcium) has been cloned (Ho, K., *et al.*, *Nature* 362:31-38 (1993)). This channel, ROMK (locus symbol KCNJ1), is the prototype of the IRK family of potassium channels, comprising two transmembrane spanning domains, and a segment homologous to the characteristic H5 pore domain. The channel contains PKA phosphorylation sites that are required for normal channel activity. Multiple ROMK isoforms encoded by the same chromosome 11 locus are generated by alternative splicing (Yano, H., *et al.*, *Mol. Pharmacology* 45:854-860 (1994); Shuck, M.E. *et al.*, *J. Biol. Chem.* 269:24261-24270 (1994)); these isoforms have been shown to be expressed in the kidney, specifically on the apical membrane of cells of the TAL as well as more distal nephron segments (Lee, W.S., *et al.*, *Am. J. Physiol.* (Renal Fluid Electrol. Physiol.) 268:F1124-31 (1995); Boim, M.A. *et al.*, *Am. J. Physiol.* (Renal Fluid Electrol. Physiol.) 268:F1132-40 (1995); Hebert, S.C., *Kidney Int.* 48, 1010-1016 (1995)). This channel has been proposed to be involved in potassium recycling in the TAL, as well as in net renal potassium secretion in the distal nephron.

The present invention provides compositions and methods that can be used to differentiate and diagnose several types of ion transport deficiencies, particularly Bartter's Syndrome and Gitelman's Syndrome. The present invention further provides methods and compositions that can be used to identify heterozygote carriers

for these disorders. Carriers, though not displaying severe clinical symptoms, nonetheless display mild to moderate pathologies.

## Summary of the Invention

The present invention is based, in part, on the identification of the roles of the human thiazide-sensitive Na-Cl cotransporter, TSC; the human ATP-sensitive $K^+$ channel, ROMK; and the human Na-K-2Cl cotransporter, NKCC2 in pathological condition associated with abnormal ion transport, particularly Bartter's Syndrome, Gitelman's Syndrome, hypokalaemic alkalosis, hypokalaemic alkalosis with hypercalciuria, kidney stones, high blood pressure, osteoporosis and sensitivity to diuretic-induced hyperkalaemia. The present invention specifically provides the amino acid sequences of several human wild-type and altered variants of the TSC, NKCC2 and ROMK proteins as well as the nucleotide sequence that encodes these variants. These proteins and nucleic acid molecules can be used in diagnosing ion transport disorders and in developing methods and agents for treating these pathologies.

## Brief Descriptions of the Drawings

Figure 1. Gitelman's Syndrome kindreds used for linkage studies. The familial relationships of Gitelman's Syndrome kindreds used for linkage studies are shown. Individuals with Gitelman's Syndrome are indicated by filled symbols; individuals who do not have Gitelman's Syndrome are indicated by unfilled symbols; deceased individuals are indicated by a diagonal line through the symbol. Individuals not sampled for genetic studies are indicated by a dot within the symbol. Each kindred is given a unique kindred number, and each individual within the kindred is numbered above and to the left of the symbol. Below each symbol, genotypes at loci on chromosome 16 are shown in their map order (see Figure 3 for map of loci). In descending order, loci shown are D16S419, D16S408, *TSC* SSCP, D16S494 and

D16S389. *TSC* SSCP refers to variants identified in *TSC*; different variants are given different allele numbers and the nature of each variant is indicated in Table 1. SSCP allele 1 represents the wild-type SSCP variant. Inferred haplotypes cosegregating with Gitelman's Syndrome are enclosed by boxes, with maternal and paternal haplotypes distinguished by shaded or unfilled boxes, respectively.

Figure 2. Characterization of the human genomic *TSC* locus.

A. Cosmid contig spanning the *TSC* locus. Thin horizontal bars represent cloned human genomic DNA of cosmid clones; vertical bars indicate sites cleaved by restriction endonuclease EcoRI. Independent clones are drawn with their overlaps indicated, and the 5' to 3' orientation of transcription is shown.

B. Intron-exon organization of the *TSC* gene. Gray boxes indicate exons of the *TSC* gene. The gene is composed of 26 coding exons, encoding a protein of 1021 amino acids. The first codon of each exon is indicated; the position in the codon of the first base of each exon is indicated by the subscript (e.g. $167_2$ indicates that the first base of exon 4 is the second base in codon 167). The exact size of each intron is not known.

C. Sequence of the human TSC protein. The sequence of the human TSC protein is shown in single letter code. The corresponding sequence of the rat and flounder TSC is shown below the human sequence; amino acids that are identical compared with the human sequence are indicated by dots, while different amino acids in the TSC of these species are indicated. The transmembrane domains proposed from hydropathy plots are shaded and numbered M1 to M12. Amino acids that are mutated on Gitelman's Syndrome alleles are highlighted, appearing in white on a black background. These variants are numbered and correspond to those indicated in Figure 1, Table 1 and Figure 5.

Figure 3. Multipoint linkage analysis of Gitelman's Syndrome and loci on chromosome 16. Multipoint linkage analysis was performed, testing for linkage of Gitelman's Syndrome to a segment of chromosome 16 containing loci D16S419,

D16S408, D16S494 and D16S389. These loci are shown in their map order, with the distance between adjacent loci indicated in centimorgans (Gyapay, G., *et al.*, *Nature Genet.* 7:246-339 (1994); Shen, Y.S., *et al.*, *Genomics* 22:68-76 (1994)). The multipoint lod score for linkage of Gitelman's Syndrome across this interval is shown, revealing a lod score of 9.5 at a recombination fraction of zero with D16S408, and showing odds of greater than 1000:1 favoring location of the trait locus in the 11 centimorgan interval defined by flanking loci D16S419 and D16S494. At the top of the figure, the lod-1 support intervals for the location of the *TSC* locus defined in CEPH kindreds and for the location of the Gitelman's locus in disease kindreds are shown, revealing that they overlap. Moreover, molecular variants in *TSC* show linkage to Gitelman's Syndrome at a recombination fraction of zero (see Figure 1).

Figure 4. Novel variants in patients with Gitelman's Syndrome. Variants in patients with Gitelman's Syndrome were identified by SSCP and subjected to DNA sequence analysis as described in Methods. Representative examples are shown. Autoradiograms of variants detected on non-denaturing gels (panels A,B,C,E,F) or denaturing gels (panel D) are shown at the left of each panel; patients are identified as in Figure 1, and subjects with Gitelman's Syndrome are indicated by asterisks; arrows indicate variants specific for Gitelman's Syndrome kindreds. At the right of each panel the DNA sequences of the corresponding variant (top) and wild-type (bottom) are shown. Variant bases are indicated by an asterisk; in panel D, the 3 bases in the wild-type sequence that are deleted in the variant are indicated by a bracket. All sequences are shown in the antisense orientation with respect to the gene. With the exception of panels D and E, 9 bases, corresponding to the mutated codon and the two flanking codons, are shown. A. Variant alters R209 to W in GIT102; B. Variant alters P349 to L in GIT107; C. Variant alters C421 to R in GIT102; D. Three base deletion changes sense sequence CCTTCA encoding PS561 to CCA, deleting codon 561 in GIT108; E. Variant in sense orientation changes consensus 3' splice site CAG to CAT in intron 15 in GIT102. F. Variant alters R955 to Q in GIT111.

Figure 5. Schematic diagram of the TSC and mutations in Gitelman's Syndrome patients. The TSC protein is represented as a 12 transmembrane domain protein with intracytoplasmic amino and carboxy termini (Gamba, G., *et al.*, *Proc. Natl. Acad. Sci. U.S.A.* 90:2749-2753 (1993); Gamba, G., *et al.*, *J. Biol. Chem.* 269:17713-17722 (1994)). The sites of mutations in exons identified in Gitelman's Syndrome patients are indicated; the numbers correspond to the numbered variants in Figure 1, 2c and Table 1. Mutations altering consensus splice sites are not shown; these are indicated in Tables 1, 3 and 4.

Figure 6. Bartter's Syndrome kindreds. Family relationships are shown. Affected subjects, unaffected subjects, living unsampled subjects and deceased subjects are indicated by filled symbols, unfilled symbols, dotted symbols and diagonal lines, respectively. Index cases are indicated by an arrow. Genotypes of loci tightly linked to *NKCC2* are indicated and are arranged in their chromosomal order (see Figure 7c); loci are identified to the left of kindred BAR152. Novel SSCP variants detected in *NKCC2* in each kindred are numbered, with the wild-type SSCP variant denoted by +. Affected offspring of consanguineous union are seen to be homozygous for all loci linked to *NKCC2*, and are homozygous for novel SSCP variants.

Figure 7. Characterization of the human genomic *NKCC2* locus.

A. Intron-exon organization. Gray boxes indicate the 26 exons encoding the NKCC2 protein. The first codon in each exon is indicated; exons that begin with the second or third base of a codon are indicated by the subscript 2 or 3, respectively.

B. Sequence of human NKCC2 protein (single letter code). The sequence of the corresponding rat and shark sequence is shown beneath the human sequence. Amino acids that are identical to human residues are indicated by dots while residues that are different in these species are indicated. Transmembrane domains proposed from hydropathy plots are shaded and numbered M1 to M12.

C. Localization of *NKCC2* on the human genetic map. Multipoint linkage analysis of *NKCC2* marker NKCGT7-3 and loci on 15q is shown. 15q loci are shown in their map order, with 15qter to the right, and the estimated genetic distance between adjacent loci in centimorgans is indicated; the lod score for linkage of *NKCC2* to each location on the map is plotted. The lod score peaks in the 3 cM interval flanked by D15S132 and D15S209, and location in this interval is supported by odds of more than 100:1 over any alternative interval.

Figure 8. Novel variants in *NKCC2* in Bartter's Syndrome patients. Variants were identified by SSCP and subjected to DNA sequence analysis. Representative examples of autoradiograms are shown at the top of each panel, and the corresponding DNA sequence of the sense strand of wild-type (left) and mutant alleles (right) are shown at the bottom of each panel. Patients are numbered as in Figure 6, and subjects with Bartter's Syndrome are indicated by asterisks. The symbol nl represents unrelated normal subjects. Arrows indicate variants specific for Bartter's Syndrome kindreds. In panels a and b, brackets above the sequence figures indicate the positions of single base insertions or deletions. In panels d and e, variant bases are indicated by an asterisk above the sequence figures. A. A single base insertion in codon ATG M195 results in a frameshift mutation. B. A single base deletion produces a frameshift in codon CGG R302. C. The last base of exon 14, representing the first base of codon 648, is mutated from G to A, changing D648 to N648. D. G to T transversion in the first base of codon 272 alters V272 to F272.

Figure 9. Principal pathways and disorders altering renal sodium reabsorption. A diagram of a nephron is shown. Plasma is filtered at the crescent-shaped structure representing the glomerulus, and sodium is reabsorbed as filtrate passes along the nephron. The physiologic mediators of sodium reabsorption are indicated, and the fraction of filtered sodium that is normally reabsorbed by each pathway is indicated. Disorders resulting from mutations in specific mediators of sodium reabsorption are indicated. The principle mediators of sodium reabsorption are: $Na^+$-$H^+$ exchange in the proximal tubule; Na-K-2Cl cotransport in the thick ascending limb of Henle; Na-

Cl cotransport in the distal convoluted tubule; electrogenic sodium reabsorption via the epithelial sodium channel, composed of at least 3 different subunits, in the distal nephron. This last pathway is indirectly coupled to secretion of $K^+$ and $H^+$.

Figure 10. Bartter's Syndrome kindreds. Family relationships are shown. Affected subjects, unaffected subjects, living unsampled subjects and deceased subjects are indicated by filled symbols, unfilled symbols, dotted symbols and diagonal lines, respectively. Index cases are indicated by an arrow. Genotypes of loci tightly linked to *NKCC2* are indicated and are arranged in their chromosomal order; these 5 loci are linked within a 3 cM interval; GT7-3 is present on the same PAC clones as *NKCC23*. Below these genotypes, novel SSCP variants detected in *ROMK* in each kindred are numbered, and correspond to numbered variants in Figure 11; the wild-type SSCP variant is denoted by +. Linkage to *NKCC2* is seen to be excluded in the consanguineous kindreds BAR159 and BAR161. In all 4 kindreds, novel *ROMK* variants are identified that cosegregate with the disease.

Figure 11. Novel variants in *NKCC2* in Bartter's Syndrome patients. Variants were identified by SSCP and subjected to DNA sequence analysis. Representative examples of autoradiograms are shown at the top of each panel, and the corresponding DNA sequence of the sense strand of wild-type (left) and mutant alleles (right) are shown at the bottom of each panel. Patients are numbered as in Figure 10, and subjects with Bartter's Syndrome are indicated by asterisks. The symbol nl represents unrelated normal subjects. Arrows indicate variants specific for Bartter's Syndrome kindreds, and are numbered as in Figure 10. In panels b and c, brackets above the sequence figures indicate the positions of base pair insertions or deletions. In panels a and d, variant bases are indicated by an asterisk above the sequence figures. A. A single base substitution changes codon TAC (Y60) to TAG (Stop60) in BAR159; this mutation is homozygous in affected, but not unaffected kindred members. B. A single base insertion produces a frameshift in codons 13-14 in BAR161; this mutation is homozygous in the affected member of this kindred. C. A 4 base deletion spanning codons 313-314 results in a frameshift mutation; the affected subject has another

missense mutation (A195V) on the other ROMK allele (data not shown). D. A single base substitution changes codon AGC (S200) to AGG (R200); this substitution eliminates a PKA phosphorylation site. The affected subject has a nonsense mutation on the other ROMK allele (W58Stop).

Figure 12. Location of ROMK mutations in patients with Bartter's Syndrome. A schematic diagram of ROMK2 is shown and depicted as spanning the plasma membrane twice with an H5 domain containing the channel pore (Ho, K., *et al.*, *Nature* 362:31-38 (1993)). The locations and consequences of mutations identified in Bartter's patients are identified.

## Modes of Carrying Out the Invention

### I. General Description

The present invention is based, in part, on the identification of the roles of the: human thiazide-sensitive Na-Cl cotransporter, TSC; the human ATP-sensitive $K^+$ channel, ROMK; and the human Na-K-2Cl cotransporter, NKCC2 in pathological conditions associated with abnormal ion transport, particularly Bartter's Syndrome, Gitelman's Syndrome, hypokalaemic alkalosis, hypokalaemic alkalosis with hypercalciuria, kidney stones, high blood pressure, osteoporosis and sensitivity to diuretic induced hyperkalaemia. The present invention specifically provides the amino acid sequences of several human wild-type and altered variants of the TSC, NKCC2 and ROMK proteins as well as the nucleotide sequence that encodes these variants. These proteins and nucleic acid molecules can be used in diagnosing ion transport disorders and in developing methods and agents for treating these pathologies.

## II. Specific Embodiments

### A. TSC, NKCC2 or ROMK Protein

Prior to the present invention the art had identified: the amino acid sequence of one allelic variant of a presumably wild-type rat and a wild-type flounder thiazide-sensitive Na-Cl cotransporter protein (TSC); the amino acid sequence of several allelic variants of a presumably wild-type human ATP-sensitive $K^+$ channel protein (ROMK); and the amino acid sequence of one allelic variant of a presumably wild-type rat, a wild-type rabbit and a wild-type mouse Na-K-2Cl cotransporter protein (NKCC2). However, prior to the present invention, no one had identified that alterations in the human variants (homologues) of these proteins result in viable individuals that suffer from pathologies caused by abnormal ion transport; no one had characterized naturally occurring human wild-type variants of the TSC and NKCC2 proteins; no one had characterized human altered variants of the TSC, ROMK and NKCC2 proteins; and no one had shown that pathological conditions that are a result of abnormal ion transport, such as Gitelman's Syndrome and Bartter's Syndrome, could be identified by analyzing a sample for the presence of a wild-type or altered variant of a TSC, NKCC2 or ROMK protein. The present invention provides, in part, the amino acid sequences of several allelic variants of wild-type human TSC protein, wild-type human NKCC2 protein, altered variants of the human TSC protein that give rise to ion transport deficiencies, and altered variants of the human NKCC2 protein that give rise to ion transport deficiencies, altered variants of the human ROMK protein that give rise to ion transport deficiencies, as well as the nucleotide sequence of the encoding nucleic acid molecules.

In one embodiment, the present invention provides the ability to produce previously unknown wild-type and altered variants of the human TSC, NKCC2 and ROMK proteins using the cloned nucleic acid molecules herein described.

As used herein, a wild-type human TSC protein refers to a protein that has the amino acid sequence of a wild-type allelic variant of human TSC. In Example 1,

DNA sequencing was performed on DNA isolated from 50 unrelated healthy individuals to identify wild-type TSC encoding DNA molecules. Figure 2 and Table 3 provide the amino acid sequences of several wild-type allelic variants of the human TSC protein. The wild-type TSC proteins of the present invention include those specifically identified and characterized herein as well as allelic variants that can be isolated and characterized without undue experimentation following the methods outlined below. For the sake of convenience, all of the wild-type human TSC proteins of the present invention will be collectively referred to as the wild-type TSC proteins or the wild-type human TSC proteins of the present invention.

The term "wild-type human TSC proteins" includes all naturally occurring allelic variants of the human TSC protein that posses normal TSC activity. In general, wild-type allelic variants of the TSC protein may/will have a slightly different amino acid sequence than that specifically provided in Seq. ID Nos ___ for the herein-described wild-type TSC proteins. Allelic variants, though possessing a slightly different amino acid sequence than those recited above, will posses the ability to transport Na, Cl, Ca, and Mg at levels equivalent to the wild-type TSC proteins herein described. Typically, allelic variants of the wild-type TSC protein will contain conservative amino acid substitutions from the wild-type TSC sequences herein described or will contain a substitution of an amino acid from a corresponding position in a TSC homologue (a TSC protein isolated from an organism other than human such as the rat or flounder homologues). Figure 2 and Table 3 identify conserved amino acid residues.

As used herein, a mutated or altered human TSC protein refers to a protein that has the amino acid sequence of a mutated or altered allelic variant of human TSC. Figure 4, Table 1 and Table 4 provide the amino acid sequences of several mutated or altered allelic variants of the human TSC protein. The mutated or altered TSC proteins of the present invention include those specifically identified and characterized herein as well as allelic variants that can be isolated and characterized without undue experimentation following the methods outlined below. For the sake

of convenience, all of the mutated or altered human TSC proteins of the present invention will be collectively referred to as the mutated or altered TSC proteins or the mutated or altered human TSC proteins of the present invention.

The term "mutated or altered human TSC proteins" includes all naturally occurring allelic variants of the human TSC protein that do not posses normal TSC activity. In general, mutated or altered allelic variants of the TSC protein may/will have a slightly to a radically different amino acid sequence than that specifically provided in Seq. ID Nos ___ for the herein-described wild-type TSC proteins. Mutated or altered allelic variants will lack or have a reduced ability to transport one or more of the ions that are transported by wild-type TSC. Typically, allelic variants of the mutated or altered TSC protein contain: non-conservative amino acid substitutions from the wild-type sequences herein described, a substitution of an amino acid other than the amino acid found in a corresponding position in a TSC homologue (a TSC protein isolated from an organism other than human), a frame shift mutation, an insertion of a stop codon, or a deletion or insertion of one or more amino acids into the TSC sequence.

As used herein, a wild-type human NKCC2 protein refers to a protein that has the amino acid sequence of a wild-type allelic variant of human NKCC2. In Example 2, DNA sequencing was performed on DNA isolated from 50 unrelated, healthy individuals to identify wild-type NKCC2 encoding DNA molecules. Figure 6 provides the amino acid sequences of the only wild-type allelic variant of the human NKCC2 protein thus far identified. Variations were seen in intron regions but no variation has been observed in the exon regions. The wild-type NKCC2 proteins of the present invention include the one specifically identified and characterized herein as well as allelic variants that can be isolated and characterized without undue experimentation following the methods outlined below. For the sake of convenience, all of the wild-type human NKCC2 proteins of the present invention will be collectively referred to as the wild-type NKCC2 proteins or the wild-type human NKCC2 proteins of the present invention.

The term "wild-type human NKCC2 proteins" includes all naturally occurring allelic variants of the human NKCC2 protein that posses normal NKCC2 activity. In general, wild-type allelic variants of the NKCC2 protein may/will have a slightly different amino acid sequence than that specifically provided in Seq. ID Nos ___ for the herein-disclosed wild-type NKCC2 proteins. Allelic variants, though possessing a slightly different amino acid sequence than those recited above, will posses the ability to transport Na, Cl, K, and Ca at levels equivalent to the wild-type NKCCS proteins herein described. Typically, allelic variants of the wild-type NKCC2 protein will contain conservative amino acid substitutions from the wild-type sequences herein described or will contain a substitution of an amino acid from a corresponding position in a NKCC2 homologue (a NKCC2 protein isolated from an organism other than human).

As used herein, a mutated or altered human NKCC2 protein refers to a protein that has the amino acid sequence of a mutated or altered allelic variant of human NKCC2. Figure 8 and Table 7 provide the amino acid sequences of several mutated or altered allelic variants of the human NKCC2 protein. The mutated or altered NKCC2 proteins of the present invention include those specifically identified and characterized herein as well as allelic variants that can be isolated and characterized without undue experimentation following the methods outlined below. For the sake of convenience, all of the mutated or altered human NKCC2 proteins of the present invention will be collectively referred to as the mutated or altered NKCC2 proteins or the mutated or altered human NKCC2 proteins of the present invention.

The term "mutated or altered human NKCC2 proteins" includes all naturally occurring allelic variants of the human NKCC2 protein that do not posses normal NKCC2 activity. In general, mutated or altered allelic variants of the NKCC2 protein may/will have a slightly to a radically different amino acid sequence than that specifically provided in Seq. ID Nos ___ for the herein-described wild-type NKCC2 proteins. Mutated or altered allelic variants will be not be able to transport one or more of the ions that are transported by wild-type NKCC2 or will transport ions at a

rate that is substantially lower than the wild-type proteins. Typically, allelic variants of the mutated or altered NKCC2 protein contain: non-conservative amino acid substitutions from a wild-type sequences herein described, a substitution of an amino acid other than the amino acid found in a corresponding position in a NKCC2 homologue (a NKCC2 protein isolated from an organism other than human), a frame shift mutation, an insertion of a stop codon, or a deletion or insertion of one or more amino acids into the NKCC2 sequence.

As used herein, a wild-type human ROMK protein refers to a protein that has the amino acid sequence of a wild-type allelic variant of human ROMK. In Example 3, DNA from 50 unrelated, healthy individuals was sequenced to identify wild-type ROMK encoding DNA molecules. The amino acid sequences of the only wild-type allelic variant of the human ROMK protein identified are disclosed in _____. Variations were seen in intron regions. However, no variation was observed in the exon regions of all ROMK encoding DNA molecules thus far examined. The wild-type ROMK proteins of the present invention include that specifically identified and characterized in the art as well as allelic variants that can be isolated and characterized without undue experimentation following the methods outlined below. For the sake of convenience, all of the wild-type human ROMK proteins of the present invention will be collectively referred to as the wild-type ROMK proteins or the wild-type human ROMK proteins of the present invention.

The term "wild-type human ROMK proteins" includes all naturally occurring allelic variants of the human ROMK protein that posses normal ROMK activity. In general, wild-type allelic variants of the ROMK protein will have a slightly different amino acid sequence than that specifically provided in Seq. ID Nos ___. Allelic variants, though possessing a slightly different amino acid sequence than those recited above, will posses the ability to be an ATP sensitive K transporter. Typically, allelic variants of the wild-type ROMK protein will contain conservative amino acid substitutions from the wild-type sequences herein described or will contain a

substitution of an amino acid from a corresponding position in a ROMK homologue (a ROMK protein isolated from an organism other than human).

As used herein, a mutated or altered human ROMK protein refers to a protein that has the amino acid sequence of a mutated or altered allelic variant of human ROMK. Figures 11 and 12 and Table 10 provide the amino acid sequences of several mutated or altered allelic variants of the human ROMK protein. The mutated or altered ROMK proteins of the present invention include those specifically identified and characterized herein as well as allelic variants that can be isolated and characterized without undue experimentation following the methods outlined below. For the sake of convenience, all of the mutated or altered human ROMK proteins of the present invention will be collectively referred to as the mutated or altered ROMK proteins or the mutated or altered human ROMK proteins of the present invention.

The term "mutated or altered human ROMK proteins" includes all naturally occurring allelic variants of the human ROMK protein that do not posses normal ROMK activity. In general, mutated or altered allelic variants of the ROMK protein may/will have a slightly to a radically different amino acid sequence than that specifically provided in Seq. ID Nos ___ for the herein-described wild-type ROMK proteins. Mutated or altered allelic variants will be not be able to transport one or more of the ions that are transported by wild-type ROMK. Typically, allelic variants of the mutated or altered ROMK protein will contain: non-conservative amino acid substitutions from the wild-type sequences herein described, a substitution of an amino acid other than the amino acid found in a corresponding position in a ROMK homologue (a ROMK protein isolated from an organism other than human), a frame shift mutation, an insertion of a stop codon, or a deletion or insertion of one or more amino acids into the ROMK sequence.

The TSC, NKCC2 and ROMK proteins of the present invention (wild-type and mutated variants) are preferably in isolated from. As used herein, a protein is said to be isolated when physical, mechanical or chemical methods are employed to remove the TSC, NKCC2 or ROMK protein from cellular constituents that are

normally associated with the protein. A skilled artisan can readily employ standard purification methods to obtain an isolated TSC, NKCC2 or ROMK protein. The nature and degree of isolation will depend on the intended use.

The cloning of TSC, NKCC2 and ROMK encoding nucleic acid molecules makes it possible to generate defined fragments of the TSC, NKCC2 and ROMK proteins of the present invention. As discussed below, fragments of the TSC, NKCC2 and ROMK proteins of the present invention are particularly useful in generating domain specific antibodies, in identifying agents that bind to a TSC, NKCC2 or ROMK protein and in identifying TSC, NKCC2 or ROMK intra- or extracellular binding partners.

Fragments of the TSC, NKCC2 and ROMK proteins can be generated using standard peptide synthesis technology and the amino acid sequences disclosed herein. Alternatively, recombinant methods can be used to generate nucleic acid molecules that encode fragments of the TSC, NKCC2 and ROMK proteins. Figures 2, 5, 7 and 12 and Tables 1, 3, 4, 7 and 10 identify amino acid residues that are altered from wild-type residues in the altered variants of the TSC, NKCC2 and ROMK1 proteins herein described. Fragments containing these residues/alterations are particularly useful in generating altered variant specific anti-TSC, NKCC2 or ROMK antibodies.

As described below, members of the TSC, NKCC2 and ROMK family of proteins can be used for, but are not limited to: 1) a target to identify agents that block or stimulate TSC, NKCC2 or ROMK activity, 2) a target or bait to identify and isolate binding partners that bind a TSC, NKCC2 or ROMK protein, 3) identifying agents that block or stimulate the activity of a TSC, NKCC2 or ROMK protein and 4) an assay target to identify TSC, NKCC2 or ROMK mediated activity or disease.

### B. Anti-TSC, NKCC2 or ROMK Antibodies

The present invention further provides antibodies that selectively bind one or more of the TSC, NKCC2 or ROMK proteins of the present invention. The most preferred antibodies will bind to an altered variant of a TSC, NKCC2 or ROMK

protein but not to a wild-type variant or will bind to a wild-type variant of a TSC, NKCC2 or ROMK protein but not to an altered variant. Anti-TSC, NKCC2 or ROMK antibodies that are particularly contemplated include monoclonal and polyclonal antibodies as well as fragments containing the antigen binding domain and/or one or more complement determining regions.

Antibodies are generally prepared by immunizing a suitable mammalian host using a TSC, NKCC2 or ROMK protein, or fragment, in isolated or immunoconjugated variant (Harlow, Antibodies, Cold Spring Harbor Press, NY (1989)). Figures 2, 5, 7 and 12 and Tables 1, 3, 4, 7 ad 10 identify several regions of the TSC, NKCC2 and ROMK proteins that have been shown to be mutated in various altered variants of the TSC, NKCC2 and ROMK proteins described herein. Fragments containing these residues are particularly suited in generating wild-type or mutated-variant specific anti-TSC, NKCC2 or ROMK antibodies.

Methods for preparing a protein for use as an immunogen and for preparing immunogenic conjugates of a protein with a carrier such as BSA, KLH, or other carrier proteins are well known in the art. In some circumstances, direct conjugation using, for example, carbodiimide reagents may be used; in other instances linking reagents such as those supplied by Pierce Chemical Co., Rockford, IL, may be effective.

Administration of the TSC, NKCC2 or ROMK immunogen is conducted generally by injection over a suitable time period and with use of a suitable adjuvant, as is generally understood in the art. During the immunization schedule, titers of antibodies can be taken to determine adequacy of antibody formation.

While the polyclonal antisera produced in this way may be satisfactory for some applications, for pharmaceutical compositions, monoclonal antibody preparations are preferred. Immortalized cell lines which secrete a desired monoclonal antibody may be prepared using the standard method of Kohler and Milstein or modifications which effect immortalization of lymphocytes or spleen

cells, as is generally known. The immortalized cell lines secreting the desired antibodies are screened by immunoassay in which the antigen is the TSC, NKCC2 or ROMK protein or peptide fragment. When the appropriate immortalized cell culture secreting the desired antibody is identified, the cells can be cultured either *in vitro* or by production in ascites fluid.

The desired monoclonal antibodies are then recovered from the culture supernatant or from the ascites supernatant. Fragments of the monoclonals or the polyclonal antisera which contain the immunologically significant portion can be used as antagonists, as well as the intact antibodies. Use of immunologically reactive fragments, such as the Fab, Fab', of $F(ab')_2$ fragments is often preferable, especially in a therapeutic context, as these fragments are generally less immunogenic than the whole immunoglobulin.

The antibodies or fragments may also be produced, using current technology, by recombinant means. Regions that bind specifically to the desired regions of the transporter can also be produced in the context of chimeric or CDR grafted antibodies of multiple species origin.

As described below, anti-TSC, NKCC2 or ROMK antibodies are useful as modulators of TSC, NKCC2 or ROMK activity, are useful in immunoassays for detecting TSC, NKCC2 or ROMK expression/activity and for purifying wild-type and altered variants of the TSC, NKCC2 and ROMK proteins.

### C. TSC, NKCC2 or ROMK Encoding Nucleic Acid Molecules

As described above, the present invention is based, in part, on isolating nucleic acid molecules from humans that encode wild-type or altered variants of the TSC, NKCC2 and ROMK proteins. Accordingly, the present invention further provides nucleic acid molecules that encode the herein disclosed wild-type and altered variants of the TSC, NKCC2 and ROMK proteins as herein defined, preferably in isolated variant. For convenience, all TSC, NKCC2 or ROMK encoding nucleic acid molecules will be referred to as TSC, NKCC2 or ROMK encoding nucleic acid

molecules, the *TSC, NKCC2 or ROMK* genes, or *TSC, NKCC2 or ROMK*. The nucleotide sequence of identified wild-type TSC encoding nucleic acid molecules are provided in Figure 2 and Table 3. The nucleotide sequence of identified altered TSC encoding nucleic acid molecules are provided in Figures 2 and 4 and Tables 1 and 4. The nucleotide sequence of identified wild-type NKCC2 encoding nucleic acid molecules are provided in Figure 7. The nucleotide sequence of identified altered NKCC2 encoding nucleic acid molecules are provided in Figure 8 and Table 7. The nucleotide sequence of identified altered ROMK encoding nucleic acid molecules are provided in Figure 11 and Table 10.

As used herein, a "nucleic acid molecule" is defined as an RNA or DNA molecule that encodes a peptide as defined above, or is complementary to a nucleic acid sequence encoding such peptides. Particularly preferred nucleic acid molecules will have a nucleotide sequence identical to or complementary to the human cDNA sequences herein disclosed. Specifically contemplated are genomic DNA, cDNA, mRNA and antisense molecules, as well as nucleic acids based on an alternative backbone or including alternative bases whether derived from natural sources or synthesized. Such nucleic acid molecules, however, are defined further as being novel and unobvious over any prior art nucleic acid molecules encoding non-human homologues of TSC, NKCC2 or ROMK isolated from non-human organisms and known human ROMK proteins.

As used herein, a nucleic acid molecule is said to be "isolated" when the nucleic acid molecule is substantially separated from contaminant nucleic acid encoding other polypeptides. A skilled artisan can readily employ nucleic acid isolation procedures to obtain an isolated TSC, NKCC2 or ROMK encoding nucleic acid molecule.

The present invention further provides fragments of the TSC, NKCC2 or ROMK encoding nucleic acid molecules of the present invention. As used herein, a fragment of a TSC, NKCC2 or ROMK encoding nucleic acid molecule refers to a small portion of the entire protein encoding sequence. The size of the fragment will be determined by

the intended use. For example, if the fragment is chosen so as to encode an active portion of the TSC, NKCC2 or ROMK protein, such an intracellular or extracellular domain, then the fragment will need to be large enough to encode the functional region(s) of the TSC, NKCC2 or ROMK protein. If the fragment is to be used as a nucleic acid probe or PCR primer, then the fragment length is chosen so as to obtain a relatively small number of false positives during probing/priming. Figures 2, 7, 11 and Tables 1-4, 6, 7, 9 and 10 identify fragments of the *TSC, NKCC2* and *ROMK* genes that are particularly useful as selective hybridization probes or PCR primers. Such fragments contain regions that are conserved among wild-type or altered variants of *TSC, NKCC2* or *ROMK*, regions of homology that are shared with the previously identified *TSC, NKCC2* and *ROMK* genes, and regions that are altered in altered variants of the *TSC, NKCC2* and *RDMK* genes.

Fragments of the TSC, NKCC2 or ROMK encoding nucleic acid molecules of the present invention (i.e., synthetic oligonucleotides) that are used as probes or specific primers for the polymerase chain reaction (PCR), or to synthesize gene sequences encoding TSC, NKCC2 and ROMK proteins, can easily be synthesized by chemical techniques, for example, the phosphotriester method of Matteucci, *et al.*; *J Am Chem Soc* (1981) 103:3185-3191 or using automated synthesis methods. In addition, larger DNA segments can readily be prepared by well known methods, such as synthesis of a group of oligonucleotides that define various modular segments of the *TSC, NKCC2 or ROMK* gene, followed by ligation of oligonucleotides to build the complete modified *TSC, NKCC2 or ROMK* gene.

The TSC, NKCC2 or ROMK encoding nucleic acid molecules of the present invention may further be modified so as to contain a detectable label for diagnostic and probe purposes. As described above, such probes can be used to identify nucleic acid molecules encoding other allelic variants of wild-type or altered TSC, NKCC2 and ROMK proteins and as described below, such probes can be used to diagnosis the presence of an altered variant of a TSC, NKCC2 or ROMK protein as a means for diagnosing a pathological condition caused by abnormal ion transport. A variety of

such labels are known in the art and can readily be employed with the TSC, NKCC2 or ROMK encoding molecules herein described. Suitable labels include, but are not limited to, biotin, radiolabeled nucleotides, biotin, and the like. A skilled artisan can employ any of the art known labels to obtain a labeled TSC, NKCC2 or ROMK encoding nucleic acid molecule.

**D. Isolation of Other Wild-Type and Altered Forms of TSC, NKCC2 and ROMK Encoding Nucleic Acid Molecules**

As described above, the identification of the role of the TSC, NKCC2 and ROMK proteins in the pathology/severity of ion transport mediated deficiencies has made possible the identification of several allelic variants of the wild-type TSC, NKCC2 and ROMK proteins as well as several altered variants of the TSC, NKCC2 and ROMK proteins that confer a pathology associated with abnormal ion transport. These observations allows a skilled artisan to isolate nucleic acid molecules that encode other wild-type and altered variants of the TSC, NKCC2 and ROMK proteins, in addition to the sequence herein described.

Essentially, a skilled artisan can readily use the amino acid sequence of the human TSC, NKCC2 and ROMK proteins to generate antibody probes to screen expression libraries prepared from cells. Typically, polyclonal antiserum from mammals such as rabbits immunized with the purified protein (as described below) or monoclonal antibodies can be used to probe a human cDNA or genomic expression library, such as lambda gtll library, prepared from a normal or effected individual, to obtain the appropriate coding sequence for wild-type or altered variants of the TSC, NKCC2 or ROMK protein. The cloned cDNA sequence can be expressed as a fusion protein, expressed directly using its own control sequences, or expressed by constructions using control sequences appropriate to the particular host used for expression of the enzyme. Figures 2, 7 and 11 and Tables 1-4, 7, 9 and 10 identify important operative domains and domains that have been shown to contain alterations in mutated variants of each of the TSC, NKCC2 and ROMK proteins. Such regions are

preferred sources of antigenic portions of the TSC, NKCC2 or ROMK protein for the production of probe, diagnostic, and therapeutic antibodies.

Alternatively, a portion of the TSC, NKCC2 or ROMK encoding sequence herein described can be synthesized and used as a probe to retrieve DNA encoding a member of the TSC, NKCC2 or ROMK family of proteins from individuals that have normal ion transport or from individuals suffering from a pathological condition that is a result of abnormal ion transport. Oligomers containing approximately 18-20 nucleotides (encoding about a 6-7 amino acid stretch) are prepared and used to screen genomic DNA or cDNA libraries to obtain hybridization under stringent conditions or conditions of sufficient stringency to eliminate an undue level of false positives. This method can be used to identify and isolate altered and wild-type variants of the TSC, NKCC2 and ROMK encoding sequences.

Additionally, pairs of oligonucleotide primers can be prepared for use in a polymerase chain reaction (PCR) to selectively amplify/clone a TSC, NKCC2 or ROMK-encoding nucleic acid molecule, or fragment thereof. A PCR denature/anneal/extend cycle for using such PCR primers is well known in the art and can readily be adapted for use in isolating other TSC, NKCC2 or ROMK encoding nucleic acid molecules. Figures 2, 7 and 11 and Tables 1-4, 6, 7, 9 and 10 identify regions of the human TSC, *NKCC2* and *ROMK* genes that are particularly well suited for use as a probe or as primers. In general, the preferred primers will flank one or more exons of the TSC, NKCC2 or ROMK encoding nucleic acid molecule.

### E. Methods for Identifying Pathological Conditions Involving Abnormal Ion Transport

The present invention further provides methods for identifying cells and individuals expressing active and altered variants of the Na-K-2Cl cotransporter NKCC2, the renal thiazide-sensitive Na-Cl cotransporter, TSC, and the ATP-sensitive potassium channel, ROMK. Such methods can be used to diagnose biological and pathological processes associated with altered ion transport, particularly various

variants of Bartter's Syndrome and Gitelman's Syndrome, the progression of such conditions, the susceptibility of such conditions to treatment and the effectiveness of treatment for such conditions. The methods of the present invention are particularly useful in identifying carriers of ion transport deficiencies, particularly Gitelman's and Bartter's Syndromes, as well as in differentiating between Gitelman's and Barrter's Syndromes. Specifically, the presence of wild-type or altered variants of the TSC, NKCC2 and ROMK proteins can be identified by determining whether a wild-type or altered variant of the TSC, NKCC2 or ROMK protein, or nucleic acid encoding one or more of these proteins, is expressed in a cell. The expression of an altered variant, or departure from the normal level of TSC, NKCC2 or ROMK expression, can be used as a means for diagnosing pathological conditions mediated by abnormal TSC, NKCC2 or ROMK activity/expression, differentiating between various ion transport deficiencies, and to identify carriers of ion transport deficiencies.

A variety of immunological and molecular genetic techniques can be used to determine if a wild-type or an altered variant of a TSC, NKCC2 or ROMK protein is expressed/produced in a particular cell and/or the level at which the protein is expressed. The preferred methods will identify whether a wild-type or mutated from of the TSC, NKCC2 or ROMK protein is expressed.

In general, an extract containing nucleic acid molecules or an extract containing proteins is prepared from cells of an individual. The extract is then assayed to determine whether a TSC, NKCC2 or ROMK protein, or a TSC, NKCC2 or ROMK encoding nucleic acid molecule, is produced in the cell. The type of protein/nucleic acid molecule expressed or the degree/level of expression provides a measurement of the nature and degree of TSC, NKCC2 or ROMK activity.

For example, to perform a diagnostic test based on nucleic acid molecules, a suitable nucleic acid sample is obtained and prepared from a subject using conventional techniques. DNA can be prepared, for example, simply by boiling the sample in SDS. Most typically, for nucleic acid samples, a blood sample, a buccal swab, a hair follicle preparation or a nasal aspirate is used as a source of cells to

provide the nucleic acid molecules. The extracted nucleic acid can then be subjected to amplification, for example by using the polymerase chain reaction (PCR) according to standard procedures, to selectively amplify a TSC, NKCC2 or ROMK encoding nucleic acid molecule or fragment thereof. The size of the amplified fragment (typically following restriction endonuclease digestion) is then determined using gel electrophoresis or the nucleotide sequence of the fragment is determined (for example, see Weber and May *Am J Hum Genet* (1989) 44:388-339; Davies, J. *et al. Nature* (1994) 371:130-136)). The resulting size of the fragment or sequence is then compared to the known wild-type, predicted wild-type, known altered variants and predicted altered variants of the protein in question. Using this method, the presence of wild-type or altered variants of the TSC, NKCC2 and ROMK proteins can be differentiated and identified.

Alternatively, the presence or absence of one or more single base-pair polymorphism(s) within the TSC, NKCC2 or ROMK encoding nucleic acid molecules can be determined by conventional methods which included, but are not limited to, manual and automated fluorescent DNA sequencing, selective hybridization probes, primer extension methods (Nikiforov, T.T. *et al. Nucl Acids Res* (1994) 22:4167-4175); oligonucleotide ligation assay (OLA) (Nickerson, D.A. *et al. Proc Natl Acad Sci USA* (1990) 87:8923-8927); allele-specific PCR methods (Rust, S. *et al. Nucl Acids Res* (1993) 6:3623-3629); RNase mismatch cleavage, single strand conformation polymorphism (SSCP) (Orita, M. *et al., Proc Natl Acad Sci USA* 86:2766-2770 (1989)), denaturing gradient gel electrophoresis (DGGE) and the like. The present diagnosis method is particularly well suit for use in biochips technologies that are being developed to be used to identify whether one of many sequence variations is present in a sample. A skilled artisan can readily adapt any nucleic acid analytical method for use in determining whether a sample contains nucleic acid molecules that encode a wild-type or altered variant of a TSC, NKCC2 or ROMK protein.

To perform a diagnostic test based on protein, a suitable protein sample is obtained and prepared from a subject using conventional techniques. Protein samples can be prepared, for example, simply by mixing the sample with SDS followed by salt precipitation of a protein fraction. Typically, for protein samples, a blood sample, a buccal swab, a nasal aspirate, or a biopsy of cells from tissues expressing a TSC, NKCC2 or ROMK protein is used as a source of cells to provide the protein molecules. The extracted protein can then be analyzed to determine the presence of a wild-type or altered variant of a TSC, NKCC2 or ROMK protein using known methods. For example, the presence of specific sized or charged variants of a protein can be identified using mobility in an electric filed. Alternatively, wild-type or altered variant specific antibodies can be used. A skilled artisan can readily adapt known protein analytical methods to determine if a sample contains a wild-type or altered variant of a TSC, NKCC2 or ROMK protein.

TSC, NKCC2 or ROMK expression can also be used in methods to identify disorders that occur as a result of an increase or decrease in the expression of a naturally occurring TSC, NKCC2 or ROMK gene. Specifically, nucleic acid probes that detect mRNA can be used to detect cells or tissues that express a TSC, NKCC2 or ROMK protein and the level of such expression.

As provided above, the presence of only an altered variant of a TSC protein (homozygous state) in a sample is diagnostic of Gitelman's Syndrome. Altered variants of the TSC protein, when present in sample that additionally contains a wild-type variant of TSC (heterozygous state), is diagnostic for carriers of Gitelman's Syndrome and individuals expressing lower levels of active TSC. Decreased levels of active TSC lead to decreased urinary calcium, increased bone density and a propensity for deposition of calcium in the joints and diuretic induced hypokalaemia. Elevated levels of TSC expression are diagnostic for increased urinary calcium, decreased bone density, and a propensity for high blood pressure and kidney stones. The presence of only an altered variant of a NKCC2 protein (homozygous state) in a sample is diagnostic of several variants of Bartter's Syndrome. Altered variants of the NKCC2 protein, when

present in sample that additionally contains a wild-type variant of NKCC2 (heterozygous state), is diagnostic for carriers of Bartter's Syndrome and individuals expressing lower levels of active NKCC2. Decreased NKCC2 activity leads to increased urinary calcium, decreased bone mass and a propensity for kidney stones, osteoporosis and diuretic induced hypokalaemia. The presence of only an altered variant of a ROMK protein (homozygous state) in a sample is diagnostic of several variants of Bartter's Syndrome. Altered variants of the ROMK protein, when present in sample that additionally contains a wild-type variant of ROMK (heterozygous state), is diagnostic for carriers of Bartter's Syndrome and individuals expressing lower levels of active ROMK. Decreased ROMK activity leads to increased urinary calcium, decreased bone mass and a propensity for kidney stones and osteoporosis.

Alternatively, TSC, NKCC2 or ROMK expression can also be used in methods to identify agents that increase or decrease the level of expression of a naturally occurring TSC, NKCC2 or ROMK gene. For example, cells or tissues expressing a TSC, NKCC2 or RMOK protein can be contacted with a test agent to determine the effects of the agent on *TSC, NKCC2 or ROMK* expression. Agents that activate *TSC, NKCC2* or *ROMK* expression can be used as an agonist of TSC, NKCC2 or ROMK activity whereas agents that decrease *TSC, NKCC2* or *ROMK* expression can be used as an antagonist of TSC, NKCC2 or ROMK activity.

### F. rDNA Molecules Containing a TSC, NKCC2 or ROMK Encoding Nucleic Acid Molecule

The present invention further provides recombinant DNA molecules (rDNAs) that contain one or more of the wild-type or altered TSC, NKCC2 or ROMK encoding sequences herein described, or a fragment of the herein-described nucleic acid molecules. As used herein, an rDNA molecule is a DNA molecule that has been subjected to molecular manipulation *in vitro*. Methods for generating rDNA molecules are well known in the art, for example, see Sambrook *et al., Molecular Cloning* (1989). In the preferred rDNA molecules, a TSC, NKCC2 or ROMK encoding DNA sequence that encodes a wild-type or altered variant of the TSC, NKCC2 or ROMK protein is

operably linked to one or more expression control sequences and/or vector sequences. Most preferably, the TSC, NKCC2 or ROMK encoding nucleic acid molecules will encode one of the novel altered or wild-type variants herein described.

The choice of vector and/or expression control sequences to which one of the TSC, NKCC2 or ROMK encoding sequences of the present invention is operably linked depends directly, as is well known in the art, on the functional properties desired, e.g., protein expression, and the host cell to be transformed. A vector contemplated by the present invention is at least capable of directing the replication or insertion into the host chromosome, and preferably also expression, of a TSC, NKCC2 or ROMK encoding sequence included in the rDNA molecule.

Expression control elements that are used for regulating the expression of an operably linked protein encoding sequence are known in the art and include, but are not limited to, inducible promoters, constitutive promoters, secretion signals, enhancers, transcription terminators and other regulatory elements. Preferably, an inducible promoter that is readily controlled, such as being responsive to a nutrient in the host cell's medium, is used.

In one embodiment, the vector containing a TSC, NKCC2 or ROMK encoding nucleic acid molecule will include a prokaryotic replicon, i.e., a DNA sequence having the ability to direct autonomous replication and maintenance of the recombinant DNA molecule intrachromosomally in a prokaryotic host cell, such as a bacterial host cell, transformed therewith. Such replicons are well known in the art. In addition, vectors that include a prokaryotic replicon may also include a gene whose expression confers a detectable marker such as a drug resistance. Typical bacterial drug resistance genes are those that confer resistance to ampicillin or tetracycline.

Vectors that include a prokaryotic replicon can further include a prokaryotic or viral promoter capable of directing the expression (transcription and translation) of the TSC, NKCC2 or ROMK encoding gene sequence in a bacterial host cell, such as *E. coli*. A promoter is an expression control element formed by a DNA sequence that

permits binding of RNA polymerase and transcription to occur. Promoter sequences compatible with bacterial hosts are typically provided in plasmid vectors containing convenient restriction sites for insertion of a DNA segment of the present invention. Typical of such vector plasmids are pUC8, pUC9, pBR322 and pBR329 available from Biorad Laboratories (Richmond, CA), pPL and pKK223 available from Pharmacia, Piscataway, NJ.

Expression vectors compatible with eukaryotic cells, preferably those compatible with vertebrate cells, can also be used to variant rDNA molecules that contain a TSC, NKCC2 or ROMK encoding sequence. Eukaryotic cell expression vectors are well known in the art and are available from several commercial sources. Typically, such vectors are provided containing convenient restriction sites for insertion of the desired DNA segment. Typical of such vectors are PSVL and pKSV-10 (Pharmacia), pBPV-1/pML2d (International Biotechnologies, Inc.), pTDT1 (ATCC, #31255), the vector pCDM8 described herein, and the like eukaryotic expression vectors.

Eukaryotic cell expression vectors used to construct the rDNA molecules of the present invention may further include a selectable marker that is effective in an eukaryotic cell, preferably a drug resistance selection marker. A preferred drug resistance marker is the gene whose expression results in neomycin resistance, i.e., the neomycin phosphotransferase (*neo*) gene. Southern *et al.*, *J Mol Anal Genet* (1982) 1:327-341. Alternatively, the selectable marker can be present on a separate plasmid, and the two vectors are introduced by cotransfection of the host cell, and selected by culturing in the presence of the appropriate drug for the selectable marker.

### G. Host Cells Containing an Exogenously Supplied TSC, NKCC2 or ROMK Encoding Nucleic Acid Molecule

The present invention further provides host cells transformed with a nucleic acid molecule that encodes a human wild-type or altered TSC, NKCC2 or ROMK protein of the present invention. The host cell can be either prokaryotic or eukaryotic. Eukaryotic

cells useful for expression of a TSC, NKCC2 or ROMK protein are not limited, so long as the cell line is compatible with cell culture methods and compatible with the propagation of the expression vector and expression of the *TSC, NKCC2* or *ROMK* gene product. Preferred eukaryotic host cells include, but are not limited to, yeast, insect and mammalian cells, preferably vertebrate cells such as those from a mouse, rat, monkey or human fibroblastic cell line, the most preferred being cells that do not naturally express a human TSC, NKCC2 or ROMK protein.

Any prokaryotic host can be used to express a TSC, NKCC2 or ROMK-encoding rDNA molecule. The preferred prokaryotic host is *E. coli*.

Transformation of appropriate cell hosts with an rDNA molecule of the present invention is accomplished by well known methods that typically depend on the type of vector used and host system employed. With regard to transformation of prokaryotic host cells, electroporation and salt treatment methods are typically employed, see, for example, Cohen *et al.*, *Proc Acad Sci USA* (1972) 69:2110; and Maniatis *et al.*, Molecular Cloning, A Laboratory Manual, Cold Spring Harbor Laboratory, Cold Spring Harbor, NY (1982). With regard to transformation of vertebrate cells with vectors containing rDNAs, electroporation, cationic lipid or salt treatment methods are typically employed, see, for example, Graham *et al.*, *Virol* (1973) 52:456; Wigler *et al.*, *Proc Natl Acad Sci USA* (1979) 76:1373-76.

Successfully transformed cells, i.e., cells that contain an rDNA molecule of the present invention, can be identified by well known techniques. For example, cells resulting from the introduction of an rDNA of the present invention can be cloned to produce single colonies. Cells from those colonies can be harvested, lysed and their DNA content examined for the presence of the rDNA using a method such as that described by Southern, *J Mol Biol* (1975) 98:503, or Berent *et al.*, *Biotech* (1985) 3:208 or the proteins produced from the cell assayed via an immunological method.

## H. Production of a TSC, NKCC2 or ROMK Protein Using an rDNA Molecule

The present invention further provides methods for producing a human wild-type or altered TSC, NKCC2 or ROMK protein that uses one of the TSC, NKCC2 or ROMK encoding nucleic acid molecules herein described. In general terms, the production of a recombinant human wild-type or altered TSC, NKCC2 or ROMK protein typically involves the following steps.

First, a nucleic acid molecule is obtained that encodes a TSC, NKCC2 or ROMK protein, such as the nucleic acid molecule depicted in Figures 2 and 7. If the TSC, NKCC2 or ROMK encoding sequence is uninterrupted by introns, it is directly suitable for expression in any host. If not, then a spliced variant of the TSC, NKCC2 or ROMK encoding nucleic acid molecule can be generated and used or the intron containing nucleic acid molecule can be used in a compatible eukaryotic expression system.

The TSC, NKCC2 or ROMK encoding nucleic acid molecule is then preferably placed in an operable linkage with suitable control sequences, as described above, to variant an expression unit containing the TSC, NKCC2 or ROMK encoding sequence. The expression unit is used to transform a suitable host and the transformed host is cultured under conditions that allow the production of the TSC, NKCC2 or ROMK protein. Optionally the TSC, NKCC2 or ROMK protein is isolated from the medium or from the cells; recovery and purification of the protein may not be necessary in some instances where some impurities may be tolerated.

Each of the foregoing steps can be done in a variety of ways. For example, the desired coding sequences may be obtained from genomic fragments and used directly in an appropriate host. The construction of expression vectors that are operable in a variety of hosts is accomplished using an appropriate combination of replicons and control sequences. The control sequences, expression vectors, and transformation methods are dependent on the type of host cell used to express the gene and were discussed in detail

earlier. Suitable restriction sites can, if not normally available, be added to the ends of the coding sequence so as to provide an excisable gene to insert into these vectors. A skilled artisan can readily adapt any host/expression system known in the art for use with TSC, NKCC2 or ROMK encoding sequences to produce a TSC, NKCC2 or ROMK protein. Particularly well suited are expression systems that result in the production of lipid vesicles containing the expressed protein. Such lipid containing vesicles are well suited for identifying agonists and antagonists of the TSC, NKCC2 or ROMK protein.

**I. Ion Transport**

As provided above, alterations in the TSC, NKCC2 or ROMK protein cause pathological conditions that are a result of abnormal ion transport. Accordingly, the wild-type and altered variants of the TSC, NKCC2 and ROMK proteins of the present invention can be used in methods to alter the extra or intracellular concentration of Na, Ca, Cl, Mg, and/or K. In general, the extra or intracellular concentration of Na, Ca, Cl, Mg, and/or K can be altered by altering the expression of a TSC, NKCC2 or ROMK protein or the activity of a TSC, NKCC2 or ROMK protein.

There are a number of situation in which it is desirable to alter the extra or intracellular concentration of Na, Ca, Cl, Mg, and/or K. Abnormal extra or intracellular pH leads to water retention, increased blood pressure, chronic respiratory and metabolic acidosis, inflammation, sperm activation/inactivation, hydroencephaly, glaucoma, colitis, etc.

Hence, a TSC, NKCC2 or ROMK protein or *TSC, NKCC2 or ROMK* gene expression can be used as a target for, or as means to alter extra or intracellular Na, Ca, Cl, Mg and/or K concentration. For example, a TSC, *NKCC2 or ROMK* gene can be introduced and expressed in cells to increase TSC, NKCC2 or ROMK expression. This provides a means and methods for altering extra and intracellular ion levels.

There are pathological conditions characterized by inappropriate extra or intracellular ion concentrations. For example, Bartter's Syndrome, Gitelman's

Syndrome, hypokalaemic alkalosis, hypokalaemic alkalosis with hypercalciuria, kidney stones, high blood pressure, osteoporosis and sensitivity to diuretic-induced hyperkalaemia, are all associated with abnormal intracellular or extracellular ion concentration. Accordingly, TSC, NKCC2 or ROMK activity/expression is targeted as a means of treating these conditions. Various methods for regulating TSC, NKCC2 or ROMK activity/expression are discussed in detail below.

### J. Identification of Agents that Bind to a TSC, NKCC2 or ROMK Protein

Another embodiment of the present invention provides methods for identifying agents that are agonists or antagonists of the TSC, NKCC2 and ROMK proteins herein described. Specifically, agonists and antagonists of a TSC, NKCC2 or ROMK protein can be first identified by the ability of the agent to bind to one of the wild-type or altered variants of the TSC, NKCC2 and ROMK proteins herein described. Agents that bind to a TSC, NKCC2 or ROMK protein can then be tested for the ability to stimulate or block ion transport in a TSC, *NKCC2 or ROMK* expressing cell.

In detail, a TSC, NKCC2 or ROMK protein is mixed with an agent. After mixing under conditions that allow association of TSC, NKCC2 or ROMK with the agent, the mixture is analyzed to determine if the agent bound the TSC, NKCC2 or ROMK protein. Agonists and antagonists are identified as being able to bind to a TSC, NKCC2 or ROMK protein.

The TSC, NKCC2 or ROMK protein used in the above assay can either be an isolated and fully characterized protein, can be a partially purified protein, can be a cell that has been altered to express a TSC, NKCC2 or ROMK protein or can be a fraction of a cell that has been altered to express a TSC, NKCC2 or ROMK protein. Further, the TSC, NKCC2 or ROMK protein can be the entire TSC, NKCC2 or ROMK protein or a specific fragment of the TSC, NKCC2 or ROMK protein. It will be apparent to one of ordinary skill in the art that so long as the TSC, NKCC2 or

ROMK protein can be assayed for agent binding, e.g., by a shift in molecular weight or change in cellular ion content, the present assay can be used.

The method used to identify whether an agent binds to a TSC, NKCC2 or ROMK protein will be based primarily on the nature of the TSC, NKCC2 or ROMK protein used. For example, a gel retardation assay can be used to determine whether an agent binds to a soluble fragment of a TSC, NKCC2 or ROMK protein whereas patch clamping, voltage clamping, ion-sensitive microprobes or ion-sensitive chromaphores can be used to determine whether an agent binds to a cell expressing a TSC, NKCC2 or ROMK protein and affects the activity of the expressed protein. A skilled artisan can readily employ numerous techniques for determining whether a particular agent binds to a TSC, NKCC2 or ROMK protein.

Once binding is demonstrated, the agent can be further tested for the ability to modulate the activity of a wild-type or altered variant of the TSC, NKCC2 or ROMK protein using a cell or oocyte expression system and an assay that detects TSC, NKCC2 or ROMK activity. For example, voltage or patch clamping, ion-sensitive microprobes or ion-sensitive chromaphores and expression in *Xenopus* oocytes or recombinant host cells can be used to determine whether an agent that binds a TSC, NKCC2 or ROMK protein can agonize or antagonize TSC, NKCC2 or ROMK activity.

As used herein, an agent is said to antagonize TSC, NKCC2 or ROMK activity when the agent reduces TSC, NKCC2 or ROMK activity. The preferred antagonist will selectively antagonize TSC, NKCC2 or ROMK, not affecting any other cellular proteins, particularly other ion transport proteins. Further, the preferred antagonist will reduce TSC, NKCC2 or ROMK activity by more than 50%, more preferably by more than 90%, most preferably eliminating all TSC, NKCC2 or ROMK activity.

As used herein, an agent is said to agonize TSC, NKCC2 or ROMK activity when the agent increases TSC, NKCC2 or ROMK activity. The preferred agonist will selectively agonize altered variants of TSC, NKCC2 or ROMK, not affecting any

other cellular proteins, particularly other ion transport proteins. Further, the preferred agonist will increase TSC, NKCC2 or ROMK activity by more than 50%, more preferably by more than 90%, most preferably more than doubling the level of TSC, NKCC2 or ROMK activity.

Agents that are assayed in the above method can be randomly selected or rationally selected or designed. As used herein, an agent is said to be randomly selected when the agent is chosen randomly without considering the specific sequences of the TSC, NKCC2 or ROMK protein. An example of randomly selected agents is the use a chemical library or a peptide combinatorial library, or a growth broth of an organism.

As used herein, an agent is said to be rationally selected or designed when the agent is chosen on a nonrandom basis which takes into account the sequence of the target site and/or its conformation in connection with the agent's action. Agents can be rationally selected or rationally designed by utilizing the peptide sequences that make up the TSC, NKCC2 or ROMK protein. For example, a rationally selected peptide agent can be a peptide whose amino acid sequence is identical to a fragment of a TSC, NKCC2 or ROMK protein.

The agents of the present invention can be, as examples, peptides, small molecules, and vitamin derivatives, as well as carbohydrates. A skilled artisan can readily recognize that there is no limit as to the structural nature of the agents of the present invention. One class of agents of the present invention are peptide agents whose amino acid sequences are chosen based on the amino acid sequence of the TSC, NKCC2 or ROMK protein.

The peptide agents of the invention can be prepared using standard solid phase (or solution phase) peptide synthesis methods, as is known in the art. In addition, the DNA encoding these peptides may be synthesized using commercially available oligonucleotide synthesis instrumentation and produced recombinantly using standard

recombinant production systems. The production using solid phase peptide synthesis is necessitated if non-gene-encoded amino acids are to be included.

Another class of agents of the present invention are antibodies immunoreactive with critical positions of the TSC, NKCC2 or ROMK protein. As described above, antibodies are obtained by immunization of suitable mammalian subjects with peptides, containing as antigenic regions, those portions of the TSC, NKCC2 or ROMK protein intended to be targeted by the antibodies. Critical regions include the domains identified in Figures 5, 7 and 12.

### K. Uses of Agents that Bind to a TSC, NKCC2 or ROMK Protein

As provided in the Background section, the TSC, NKCC2 and ROMK proteins are involved in regulating intracellular and extracellular ion concentration. Agents that bind a TSC, NKCC2 or ROMK protein and act as an agonist or antagonist can be used to modulate biological and pathologic processes associated with TSC, NKCC2 or ROMK function and activity. In detail, a biological or pathological process mediated by TSC, NKCC2 or ROMK can be modulated by administering to a subject an agent that binds to a TSC, NKCC2 or ROMK protein and acts as an agonist or antagonist of TSC, NKCC2 or ROMK activity.

As used herein, a subject can be any mammal, so long as the mammal is in need of modulation of a pathological or biological process mediated by TSC, NKCC2 or ROMK. The term "mammal" means an individual belonging to the class Mammalia. The invention is particularly useful in the treatment of human subjects.

As used herein, a biological or pathological process mediated by TSC, NKCC2 or ROMK refers to the wide variety of cellular events mediated by a TSC, NKCC2 or ROMK protein. Pathological processes refer to a category of biological processes which produce a deleterious effect. For example, pathological processes mediated by TSC, NKCC2 or ROMK include hypokalaemic alkalosis, hypokalaemic alkalosis with hypercalciuria, kidney stones, high blood pressure, osteoporosis and sensitivity to diuretic-induced hyperkalaemia. These pathological processes can be

modulated using agents that reduce or increase the activity of a TSC, NKCC2 or ROMK protein. Preferably, the agent will act to activate an otherwise inactive altered variant of a TSC, NKCC2 or ROMK protein.

As used herein, an agent is said to modulate a pathological process when the agent reduces the degree or severity of the process. For example, an agent is said to modulate Bartter's Syndrome when the agent contributes to normal intra and extracellular ion concentrations.

## L. Administration of Agonists and Antagonists of a TSC, NKCC2 or ROMK Protein

Agonists and antagonists of the TSC, NKCC2 or ROMK protein can be administered via parenteral, subcutaneous, intravenous, intramuscular, intraperitoneal, transdermal, or buccal routes. Alternatively, or concurrently, administration may be by the oral route. The dosage administered will be dependent upon the age, health, and weight of the recipient, kind of concurrent treatment, if any, frequency of treatment, and the nature of the effect desired. For example, to treat pathological conditions resulting from abnormal ion transport, such as water retention, increased blood pressure, chronic respiratory and metabolic acidosis, inflammation, etc., an agent that modulates TSC, NKCC2 or ROMK activity is administered systemically or locally to the individual being treated. As described below, there are many methods that can readily be adapted to administer such agents.

The present invention further provides compositions containing an antagonist or agonist of a TSC, NKCC2 or ROMK protein that is identified by the methods herein described. While individual needs vary, a determination of optimal ranges of effective amounts of each component is within the skill of the art. Typical dosages comprise 0.1 to 100 μg/kg body wt. The preferred dosages comprise 0.1 to 10 μg/kg μg/kg body wt. The most preferred dosages comprise 0.1 to 1 μg/kg body wt.

In addition to the pharmacologically active agent, the compositions of the present invention may contain suitable pharmaceutically acceptable carriers

comprising excipients and auxiliaries which facilitate processing of the active compounds into preparations which can be used pharmaceutically for delivery to the site of action. Suitable formulations for parenteral administration include aqueous solutions of the active compounds in water-soluble variant, for example, water-soluble salts. In addition, suspensions of the active compounds and as appropriate, oily injection suspensions may be administered. Suitable lipophilic solvents or vehicles include fatty oils, for example, sesame oil, or synthetic fatty acid esters, for example, ethyl oleate or triglycerides. Aqueous injection suspensions may contain substances which increase the viscosity of the suspension and include, for example, sodium carboxymethyl cellulose, sorbitol, and/or dintran. Optionally, the suspension may also contain stabilizers. Liposomes can also be used to encapsulate the agent for delivery into the cell.

The pharmaceutical formulation for systemic administration according to the invention may be formulated for enteral, parenteral or topical administration. Indeed, all three types of formulations may be used simultaneously to achieve systemic administration of the active ingredient.

Suitable formulations for oral administration include hard or soft gelatin capsules, pills, tablets, including coated tablets, elixirs, suspensions, syrups or inhalations and controlled release variants thereof.

**M. Combination Therapy**

The agents of the present invention that modulate TSC, NKCC2 or ROMK activity can be provided alone, or in combination with another agents that modulate a particular biological or pathological process. For example, an agent of the present invention that reduces TSC, NKCC2 or ROMK activity can be administered in combination with other agents that affect the target ion transporter, for example, a diuretic agent. As used herein, two agents are said to be administered in combination when the two agents are administered simultaneously or are administered independently in a fashion such that the agents will act at the same time.

## N. Animal Models and Gene Therapy

The *TSC, NKCC2* and *ROMK* genes and the TSC, NKCC2 and ROMK proteins can also serve as targets for gene therapy in a variety of contexts. For example, in one application, TSC, NKCC2 or ROMK-deficient non-human animals can be generated using standard knock-out procedures to inactivate a TSC, *NKCC2 or ROMK* gene or, if such animals are non-viable, inducible TSC, NKCC2 or ROMK antisense molecules can be used to regulate TSC, NKCC2 or ROMK activity/expression. Alternatively, an animal can be altered so as to contain a TSC, NKCC2 or ROMK or antisense-TSC, NKCC2 or ROMK expression unit that directs the expression of TSC, NKCC2 or ROMK or the antisense molecule in a tissue specific fashion. In such uses, a non-human mammal, for example a mouse or a rat, is generated in which the expression of the *TSC, NKCC2 or ROMK* gene is altered by inactivation or activation. This can be accomplished using a variety of art-known procedures such as targeted recombination. Once generated, the TSC, NKCC2 or ROMK-deficient animal, the animal that expresses TSC, NKCC2 or ROMK in a tissue specific manner, or an animal that expresses an antisense molecule can be used to 1) identify biological and pathological processes mediated by TSC, NKCC2 or ROMK, 2) identify proteins and other genes that interact with TSC, NKCC2 or ROMK, 3) identify agents that can be exogenously supplied to overcome TSC, NKCC2 or ROMK deficiency and 4) serve as an appropriate screen for identifying mutations within *TSC, NKCC2 or ROMK* that increase or decrease activity.

For example, it is possible to generate transgenic mice expressing the human minigene for TSC, NKCC2 or ROMK in a tissue specific-fashion and test the effect of over-expression of the protein in cells and tissues that normally do not contain TSC, NKCC2 or ROMK. This strategy has been successfully used for other proteins, namely *bcl-2* (Veis *et al. Cell* 75:229 (1993)). Such an approach can readily be applied to the TSC, NKCC2 or ROMK protein and can be used to address the issue of a potential beneficial effect of TSC, NKCC2 or ROMK in a specific tissue area.

In another embodiment, genetic therapy can be used as a means for modulating a TSC, NKCC2 or ROMK-mediated biological or pathological processes. For example, it may be desirable to introduce into a subject being treated a genetic expression unit that encodes a modulator of TSC, NKCC2 or ROMK expression, such as an antisense encoding nucleic acid molecule or a TSC, NKCC2 or ROMK encoding nucleic acid molecule, or a functional TSC, NKCC2 or ROMK expression unit. Such modulators can either be constitutively produced or inducible within a cell or specific target cell. This allows a continual or inducible supply of a modulator of TSC, NKCC2 or ROMK or the protein expression within a subject.

The following examples are intended to illustrate, but not to limit, aspects of the present invention.

## EXAMPLE 1

Inherited hypokalaemic alkalosis with hypocalciuria (Gitelman's form of Bartter's Syndrome) is caused by mutations in the thiazide-sensitive Na-Cl cotransporter

Methods

Recruitment of Gitelman's Syndrome Kindreds. Eleven of the recruited Gitelman's Syndrome kindreds have been previously reported (Iwata, F., *et al.*, *Acta Paed. Japonica* 35:252-257 (1993); Marco-Franco, J.E., *et al.*, *Clin. Neph.* 42:33-37 (1994); Zarraga Larrondo, S., *et al.*, *Nephron* 62:340-344 (1992); Smilde, TJ., *et al.*, J. of *Rheum.* 21:1515-1519 (1994); Okusa, M.D. and Bia, M.J. Bartter's Syndrome In: Hormone Resistance and Other Endocrine Paradoxes (eds. Cohen, P. and Foa, P.) 231-263 (Springer Verlag, New York, 1987); Gitelman, H.J., *Trans. Assoc. Am. Phys.* 79:221-235 (1966); Cushner, H.M., *et al.*, *Amer. J. Kid. Dis.* 16:495-500 (1990); Sutton, R.A.L., *et al.*, *Miner. Elect. Metab.* 18:43-51 (1992)). The remaining kindred, GIT102, was ascertained through an index case referred for evaluation of

hypokalaemic metabolic alkalosis. Genomic DNA was prepared from venous blood of members of Gitelman's Syndrome kindreds by standard procedures (Bell, G., *et al.*, *Proc. Natl. Acad. Sci. USA* 78:5759-5763 (1981)).

Cloning and Characterization of the Human Genomic *TSC*. Partial human and mouse cDNAs encoding the TSC protein were isolated by PCR using oligonucleotide primers corresponding to either flounder (Gamba, G., *et al.*, *Proc. Natl. Acad. Sci. U.S.A.* 90:2749-2753 (1993)) or rat (Gamba, G., *et al.*, *J. Biol. Chem.* 269:17713-17722 (1994)) *TSC* cDNA sequence and either mouse kidney cDNA (Obermüller, N., *et al.*, *Amer. J. Physiol.*, in press (1995)) or a human kidney cDNA library as template. The resulting cDNA segments were radiolabeled (Feinberg, A.P., *et al.*, *Anal. Biochem.* 132:6-13 (1983)) and used to screen a human genomic cosmid library by hybridization; the library and screening procedures have been described previously (Shimkets, R.A., *et al.*, *Cell* 79:407-414 (1994)). Maps of resulting clones were defined by the overlapping products of digestion as well as by hybridization to specific exon or cDNA segments. Genomic fragments bearing exons of *TSC* were subcloned by digestion of cosmid DNA to completion with Sau3AI and ligation of products into BamHI-digested pBluescript; resultant clones hybridizing to mouse or human *TSC* cDNA were isolated and subjected to DNA sequence analysis by the dideoxy chain termination method using an ABI 373 instrument and following a standard protocol.

Marker Development, Genotyping, and Linkage Analysis. Sau3A1 subclones of the cosmid contig were screened for (GT)n repeat sequences by hybridization as previously described (Shimkets, R.A., *et al.*, *Cell* 79:407-414 (1994)). This screen identified clone phTSCGT-14; sequence analysis revealed an interrupted array of (GT) (Clive, D.M. *Am. J. Kid. Dis.* 25:813-823 (1995)); primers flanking this simple sequence repeat were designed and used to direct PCR from genomic DNA of unrelated subjects (primer hTSCGT14-A: 5'-GTGAGCCACTGCGCTTAGCTG-3'; hTSCGT14-B: 5'-CTGCTGAGCTCTGGTCTGGAG-3').

Genotyping of CEPH and disease kindreds for loci indicated in the text was performed by polymerase chain reaction using specific primers (Gyapay, G., *et al.*, *Nature Genet.* 7:246-339 (1994), Shen, Y.S., *et al.*, *Genomics* 22:68-76 (1994)) as described previously (Shimkets, R.A., *et al.*, *Cell* 79:407-414 (1994)), except that the products were labeled by inclusion of 1 μCi of [I-$^{32}$P]dCTP in the reaction mixture. All genotypes were scored independently by two investigators who were blinded to affection status.

Analysis of linkage was performed using the LINKAGE programs (Lathrop, G.M., *et al.*, *Proc. Natl. Acad. Sci. USA* 81:3443-3446 (1984)). Gitelman's Syndrome was specified as an autosomal recessive trait with 99% penetrance, a sporadic prevalence of 0.001, and a mutant allele frequency of 1 in 200.

SSCP and DNA Sequencing. Single-strand conformational polymorphism (Orita, M., *et al.*, *Proc. Natl. Acad. Sci. USA* 86:2766-2770 (1989)) was performed by using 27 sets of specific primers (Table 2) to separately direct PCR using genomic DNA of disease family members or CEPH controls as a template as previously described (Shimkets, R.A., *et al.*, *Cell* 79:407-414 (1994)). Amplified products were analyzed for molecular variants by electrophoresis under 3 different non-denaturing conditions as well as under denaturing urea gels as previously described (Shimkets, R.A., *et al.*, *Cell* 79:407-414 (1994)). Identified variants were eluted from the gels, reamplified by PCR and sequenced using an ABI 373 instrument as previously described (Shimkets, R.A., *et al.*, *Cell* 79:407-414 (1994)). In all cases, DNA sequences were confirmed by sequencing both DNA strands.

Results

Multiplex Gitelman's Syndrome kindreds

Thirty patients with Gitelman's Syndrome from 12 unrelated families were recruited for the initial study; 11 of these are multiplex families, with 2 or more affected subjects, and 11 of these families have been reported previously (see Methods). In 8 families, samples were obtained from two or more affected members

(Figure 1), and in 4 families a single affected subject was sampled. The diagnosis of hypokalaemic metabolic alkalosis was based on the finding of spontaneous hypokalaemia (serum potassium < 3.0 meq/l; nl > 3.5 meq/l), with metabolic alkalosis (serum bicarbonate > 29 meq/l, nl < 26 meq/l) in the absence of intercurrent illness, hypertension and diuretic therapy. All patients studied had elevated plasma renin activity. Diuretic abuse was excluded by the absence of detectable diuretics in urine.

Gitelman's Syndrome was prospectively distinguished from Bartter's Syndrome following the criteria of Bettinelli (Bettinelli, A., *et al.*, *J. Pediatr.* 120:38-43 (1992)): index cases in all of these families had marked hypocalciuria (<2 mg/kg/day, nl >4 mg/kg/day), hypomagnesemia (<0.5 meq/l, nl 0.8-1.0 meq/l), and symptomatic presentation after age 8. Twenty-seven patients were symptomatic, presenting with a variety of muscular symptoms including persistent muscular weakness, recurrent cramping with exertion, carpopedal spasm, total body tetany, and periodic paralysis with respiratory compromise. Other manifestations included seizures, paresthesias, polyuria/polydipsia, and joint pains attributable to chondrocalcinosis. Subjects classified as unaffected within multiplex families were over age 20, clinically asymptomatic, and had normal serum potassium and magnesium levels. All diagnoses were established prospectively. Affected subjects were the offspring of two clinically normal parents, consistent with recessive inheritance, with the exception of kindred GIT112, in which the disease is present in 2 successive generations; in addition, in kindred GIT102 the disease is found in second cousins, but is absent in the relatives linking these individuals (Figure 1).

Cloning and characterization of the human *TSC*. To permit testing *TSC* as a candidate gene for Gitelman's Syndrome, we identified genomic cosmid clones encoding the human homolog of TSC by hybridization to mouse or flounder *TSC* cDNAs (see Methods). Five resulting clones were characterized, with their maps revealing that they overlap and define a single contig of 55 kb (Figure 2a). The intron-exon organization of the gene was determined, demonstrating that the human TSC protein is encoded by 26 exons (Figure 2b, 2c); all intron-exon boundaries have

conventional 5' GT and 3' AG consensus splice sites. The encoded human protein shows 89% identity with rat TSC and 63% identity with flounder TSC (Figure 2c). The human protein contains 17 amino acids that are not present in the rat; this additional segment is encoded in a separate exon, exon 20. Southern blotting indicates the presence of a single gene encoding this protein in the human genome (data not shown).

A polymorphic genetic marker (TSCGT-1) at this locus was found by identification of a GT dinucleotide repeat sequence in the contig. Amplification of this segment from genomic DNA of unrelated subjects using specific primers (see Methods) demonstrated that this marker is polymorphic, displaying 3 alleles and showing a heterozygosity of 48% in 45 unrelated Caucasian subjects. This marker was shown to be linked to the *TSC* locus by finding this same marker on 4 independent cosmids from the cloned contig, as well as by subsequent linkage analysis of *TSC* variants with this marker in disease families (data not shown).

This marker was used to localize *TSC* on the human genetic map by linkage in CEPH pedigrees. Pairwise analysis revealed strong evidence for linkage to a cluster of markers on human chromosome 16. Multipoint linkage analysis confirmed linkage to chromosome 16, with a peak lod score of 10.1 in the 3 cM interval flanked by D16S408 and D16S494, with odds favoring location in this interval of 950 to 1 over the next most likely interval (Figure 3). These findings localize the *TSC* on the human genetic map and identify highly informative genetic markers spanning the *TSC* locus, permitting us to test for linkage between this segment of chromosome 16 and Gitelman's Syndrome.

Linkage of Gitelman's Syndrome and *TSC*. Highly informative markers spanning the *TSC* locus were genotyped in multiplex Gitelman's Syndrome kindreds (Figure 1). Linkage was analyzed under a prospectively specified model of recessive inheritance (see Methods). Pairwise linkage analysis revealed a maximum lod score of 6.3 for linkage of Gitelman's Syndrome and locus D16S408 at a recombination fraction of zero. Multipoint linkage analysis (Figure 3) confirmed strong evidence for

linkage, demonstrating a maximum lod score of 9.5 for linkage of Gitelman's Syndrome and D16S408 at a recombination fraction of zero (odds of more than 3 billion to 1 in favor of linkage). Flanking markers demonstrated recombinants with Gitelman's Syndrome (Figure 1 and Figure 3), localizing the gene with odds of greater than 1000:1 to the 11 centimorgan interval flanked by loci D16S419 and D16S494. Within this interval, the lod-1 support interval localizes the gene to a 7 cM segment that includes the location of the *TSC* gene (Figure 3). These findings reveal no recombinants between Gitelman's Syndrome and the segment of chromosome 16 containing *TSC*, and are consistent with both genetic homogeneity of the trait and autosomal recessive transmission in all families studied.

Mutations in *TSC* in Gitelman's Syndrome patients. The finding of complete linkage of *TSC* and Gitelman's Syndrome motivated the search for mutations in this gene in affected subjects. Twenty-seven pairs of specific primers were used to amplify 150-300 base pair segments of exons and intron-exon boundaries of the gene by polymerase chain reaction, using genomic DNA of affected subjects as a template as described in Methods; these primer sets cover the coding region and splice sites of the gene. Variants were identified by single strand conformational polymorphism (SSCP) and were subjected to DNA sequence analysis (Figure 4).

Seventeen different molecular variants inferred to alter the structure of the encoded protein were identified on the 26 mutant alleles (Tables 1 and 4, Figure 4), while no such variants were detected on 26 alleles from control subjects. Three variants were found to be homozygous in Gitelman's patients, with inheritance from both parents (Tables 1 and 4). All of these variants cosegregated with the disease in multiplex families (Figure 1) and multipoint linkage of Gitelman's Syndrome versus D16S408, *TSC* variants and D16S494 yields a lod score of 10.6 at a recombination fraction of zero with both *TSC* and D16S408 (data not shown). None of these specific variants were detected in any of 80 alleles from unrelated Caucasian control subjects.

These 17 variants are distributed throughout the gene (Figure 2c, Figure 5). Thirteen of these variants are missense (Tables 1 and 4), and these all alter residues

that are identical in normal humans and flounder (Figure 2C), species that last shared a common ancestor 400 million years ago. There is a strong bias for non-conservative amino acid substitutions (11/13 missense variants); 7 of these alter the charge of the encoded amino acid, and 2 introduce or remove a proline residue (Tables 1 and 4). Two other variants alter splice site consensus sequences, changing the CAG 3' consensus splice sequence to CAT at the junction of intron 15 and exon 16 (variant #5 in GIT102, Tables 1 and 4), and changing GT to TT at the junction of exon 24 and intron 24 (#13 in GIT105). Both of these splice site variants are highly unlikely to give rise to normal proteins. One variant (#7 in GIT108) deletes 3 base pairs, resulting in deletion of a serine residue at codon 561; this serine residue is a potential protein kinase C phosphorylation site in the cytoplasmic carboxy terminus of the protein (Figure 2c). Finally, one variant (#10 in GIT112) introduces a premature termination codon, deleting the last 54 amino acids from the encoded protein.

**Table 1. Mutations in the thiazide-sensitive Na-Cl cotransporter gene (TSC) in Gitelman's Syndrome**

| Kindred | Location | Mutation | Consequence | Homozygous | Variant # |
|---|---|---|---|---|---|
| GIT100 | Spain | CTT → CCT | L850P | + | 2 |
| GIT102 | USA | TGC → CGC | C421R | - | 3 |
| | | CGG →TGG | R209W | - | 4 |
| | | CAG →CAT | Intron 15 3' splice site | - | 5 |
| GIT103 | USA | CGC → CAC | R655H | - | 17 |
| GIT104 | USA | CGC → CTC | R653L | - | 12 |
| GIT105 | USA | CGT →GTT | Intron 24 5' splice site | - | 13 |
| GIT106 | Spain | GCG → GTG | A588V | - | 18 |
| GIT107 | England | CCC →CTC | P349L | + | 6 |
| GIT108 | Sweden | CC**TTC**A → CCA | PS561P | - | 8 |
| | | GGC → GTC | G630V | - | 14 |
| GIT109 | Canada | GAC → AAC | D486N | - | 14 |
| GIT110 | Philippines | GCA → ACA | A728T | - | 15 |
| | | GGC → TGC | G496C | - | 16 |
| GIT111 | Japan | CGG → CAG | R955Q | + | 9 |
| GIT112 | Netherlands | CGA → TGA | R968stop | - | 10 |
| | | GGG → AGG | G741R | - | 11 |

Sequences of the sense strand of the gene are shown. Underlined sequences indicate consensus splice sites at intron-exon junctions; bold sequence in GIT108 indicates segment deleted in variant. Variant # refers to variant numbers in Figs 1 and 5.

Table 2. PCR Primers for SSCP analysis of *TSC*

| Primer name | Exon | Forward primer | Reverse primer |
|---|---|---|---|
| hTSCex1A | 1 | TCCTGGCCCCTCCCTGGACAC | ATAGAGCTCATATGTGGGCAC |
| hTSCex1B | 1 | CAGCACCTTCTGCATGCGCAC | GGAAGTGGCCAGTCTTCTGAG |
| hTSCex2 | 2 | CTACCTGCCTGACTTGTGGTC | TCGACATCACGCACCACCCAC |
| hTSCex3 | 3 | TGTCCACCCAGGTGGCCTCTG | GCTGGGAAGAATGGGATTCAG |
| hTSCex4 | 4 | GCCCTGCCTAAGCTTTGGGTG | CTCGAGAGGAGGGCCTTGGTG |
| hTSCex5 | 5 | TGGTTTCATGGTTCCCGGCTC | ATCCCTCTACCCAGGGTCCAG |
| hTSCex6 | 6 | CAGAGGGTGGCTTGCAGCCTG | GCTTCTCCACGTGACCACCTC |
| hTSCex7 | 7 | TACTGACCTCTGAGGTCCTTC | AGAGCCATGGTCAGGGCCTTG |
| hTSCex8 | 8 | AGTCTTACTCATCAGGCCTTG | CGGCAGATGCCACTAGAGCAG |
| hTSCex9 | 9 | CTCTCTCCCTCCCTCCTTCAG | CTGCAGGGTGGAGGCCAGGTC |
| hTSCex10 | 10 | AGGACAGAGTAAGGAGGGAAG | GTGTCTGGTGGGTCAGCTCTC |
| hTSCex11 | 11 | CAGTAGGGAATGAAGTGCCAC | TTGTGCCTCTAGCCCAGGCTC |
| hTSCex12 | 12 | AGTGGCAGGTCCCAGCCTAAG | AACAGGAGGCCAGGCCCTGTG |
| hTSCex13 | 13 | AGACTGTCCTCTCTCTCCCTG | TGCCTCCTCCTGAGGTGGGTG |
| hTSCex14 | 14 | AGGCATGCCCACTGACTGGTG | GCCGCCTGCATGGCTACCCTG |
| hTSCex15 | 15 | CGTGTCTGGTTTCCTCTAGTG | GTGGAGCCATCACTGGCCCTG |
| hTSCex16 | 16 | AGGTGCCTTTCGCACCCAGAC | TGCTGGGTTTACAGGCATGAG |
| hTSCex17 | 17 | GACATCACCAGCTGCCTTCAC | GCCACCAAGCCGTAAGTCCTG |
| hTSCex18 | 18 | GTTCCCCATCTCACCCCTATC | CACTTGCTCAAGGCCCAATGG |
| hTSCex19 | 19 | GGAGAAGCTGGACCTCACCTC | AGAACTTTCTGGGAGTGGGTG |
| hTSCex20 | 20 | ACGGTGCCCTCAGACAAGGAG | GAGTGCCCTGAGCTCTGAGTG |
| hTSCex21 | 21 | GGCTGCTGGCTCTGCTCTGAC | GGGCAGGAGGGCTGATCCAAG |
| hTSCex22 | 22 | CATAGTGCTCTGTCCTGAGTG | AGATGACACTGGTCCCTGCAG |
| hTSCex23 | 23 | GACAGAGCAAGACGCTGTCTC | CACAGTTGGCCCTTCTGCCTG |
| hTSCex24 | 24 | TCTCAGCCGGCCTCAACCCAC | TCCCTGACCCAGTGATGTGTC |
| hTSCex25 | 25 | CGTGAAGGATTGAGTGACCTC | CACCTGACTCTGGACAGACTG |
| hTSCex26 | 26 | ACTTTGCCCATAGGGAGGAAG | AGAGCTGTGGACAGGGATGTC |

Primers (5'-3') are all within introns with the exception of: hTSCex1A-reverse and hTSCex1B-forward, which lie within exon 1; hTSCex26-forward lies at the beginning of exon 26 and hTSCex26-reverse lies distal to the normal termination codon.

Table 3. Normal variants in the Na-Cl cotransporter TSC

| Codon | Normal sequence 1 | Normal sequence 2 | Consequences of Variant |
|---|---|---|---|
| 1017 | ATC | ATT | $I_{1017}I$ |
| 499 | TAC | TAT | $Y_{499}Y$ |
| 875 | GGC | GGT | $G_{875}G$ |
| 628 | TCG | TCA | $S_{628}S$ |
| 714 | GCC | GCT | $A_{714}A$ |
| 913 | CGG | CAG | $R_{913}Q$ |

DNA sequence of each codon is shown. The consequence of each variant sequence on the encoded protein is shown. $I_{1017}I$ denotes that the substitution of ATT for ATC does not change the isoleucine residue encoded at position 1017.

Table 4. Summary of mutations in *TSC* that cause Gitelman's Syndrome

| PHENOTYPE | MUTATION | CONSEQUENCE | HOMOZ. |
|---|---|---|---|
| GITELMAN | CTT → CCT | L859 → P | + |
| | | | |
| GITELMAN | TGC → CGC | C421 → R | |
| | CGG → TGG | R209 → W | |
| | CAG → CAT | Intron 15 - 3' splice | |
| GITELMAN | CGC → CAC | R655 → H | |
| | AAG → AGG | K284 → R | |
| GITELMAN | CGC → CTC | R655 → L | |
| | | | |
| GITELMAN | GGT → GTT | Intron 24 - 5' splice | |
| | CGG → CAG | R964 → Q | |
| GITELMAN | GCG → GTG | A588 → V | |
| | CTT → CCT | L859 → P | |
| GITELMAN | CCC → CTC | P349 → L | + |
| | | | |
| GITELMAN | CCTTCA → CCA | PS561 → P | |
| | GGC → GTC | G630 → V | |
| GITELMAN | CAC → AAC | D486 → N | |
| | CGT → TGT | R928 → C | |
| GITELMAN | GCA → ACA | A728 → T | |
| | GGC → TGC | G496 → C | |
| | | | |
| GITELMAN | CGG → CAG | R964 → Q | + |
| | | | |
| GITELMAN | CGA → TGA | R977 → TERM | |
| | GGG → AGG | G741 → R | |
| | CGT → TGT | R928 → C | |
| GITELMAN | CGC → UGC | R861 → C | + |
| | | | |
| GITELMAN | UGC → UAC | C994 → Y | |
| | | | |
| GITELMAN | GGG → AGG | G741→ R | |
| | | | |
| GITELMAN | GGT → GTT | Intron 9 - 5' splice | + |
| | | | |
| GITELMAN | GGG → AGG | G741 → R | + |
| | | | |
| GITELMAN | GGC → AGC | G439 → S | |

| PHENOTYPE | MUTATION | CONSEQUENCE | HOMOZ. |
|---|---|---|---|
| | CAC → AAC | H69 → N | |
| GITELMAN | CGG → CAG | R964 → Q | |
| | | | |
| GITELMAN | GGC → AGC | G439 → S | + |
| | | | |
| GITELMAN | CGG → CAG | R964 → Q | |
| | AAG → AAC | K734 → N | |
| GITELMAN | 7 bp deletion | H696 → FS | |
| | GGT → GTT | G729 → V | |
| GITELMAN | AGG → TGG | R334 → W | |
| | | | |
| GITELMAN | 2 bp deletion | G731 → FS | |
| | GGC → AGC | G439 → S | |
| GITELMAN | 1 bp deletion | F765 → FS | |
| | | | |
| GITELMAN | AGG → TGG | R334 → W | |
| | CGT → TGT | R399 → C | |
| GITELMAN | CGC → TGC | R861 → C | |
| | | | |
| GITELMAN | GGG → AGG | G741 → R | |
| | CGC → CAC | R642 → H | |
| | | | |
| GITELMAN | GGT → GTT | intron 9 - 5' splice | + |
| | | | |
| GITELMAN | 7 bp insertion | D400 → TERM | |
| | GGC → GAC | G460 → D | |
| GITELMAN | CGT → CTT | R399 → L | |
| | | | |
| GITELMAN | CGG → TGG | R321 → W | |
| | CTT → CCT | L859 → P | |
| GITELMAN | CGG → CAG | R209 → Q | |
| | AAA → UAA | K497 → TERM | |
| GITELMAN | CGC → GGC | R642 → G | |
| | AGC → ATC | S448 → I | |
| GITELMAN | AUG → AAG | M343 → K | + |
| | | | |
| GITELMAN | GGG → AGG | G741 → R | + |
| | | | |
| GITELMAN | GTG → ATG | V1024 → M | |
| | ATG → AAG | M581 → K | |
| GITELMAN | GGG → AGG | G741 → R | |
| | | | |

| PHENOTYPE | MUTATION | CONSEQUENCE | HOMOZ. |
|---|---|---|---|
| GITELMAN | CGG → CAG | R964 → Q | |
| | GAC → AAC | D486 → N | |
| GITELMAN | GGG → AGG | G741 → R | |
| | | | |
| GITELMAN | CCC → CTC | P643 → L | |
| | CGG → CAG | R964 → Q | |
| GITELMAN | CTT → CCT | L859 → P | |
| | CGT → CTT | R399 → L | |
| GITELMAN | CGC → CAC | R655 → H | |
| | GT → AT | Intron 5 - 5'SPLICE | |
| GITELMAN | CCC → CTC | P643 → L | |
| | | | |
| GITELMAN | GGG → AGG | G989 → R | |
| | | | |
| GITELMAN | TCC → CCC | S620 → P | |
| | | | |
| GITELMAN | GAC → AAC | D486 → N | |
| | | | |
| GITELMAN | CGG → CAG | R964 → Q | |
| | | | |
| GITELMAN | CGT → GT | R928 → C | |
| | | | |
| GITELMAN | CAG → TAG | E112 → TERM | |
| | GGT → GTT | G729 → V | |
| GITELMAN | GGT → GTT | Intron 9 - 5' splice | "+" |
| | | | |
| GITELMAN | GGT → GTT | Intron 24 - 5' splice | "+" |
| | | | |
| GITELMAN | CGC → TGC | R861 → C | |
| | CGA →CAA | R896 → Q | |
| GITELMAN | CGC → TGC | R861 → C | |
| | | | |
| GITELMAN | 7 BP DELETION | AA 969 | |
| | | | |
| GITELMAN | CGA → TGA | R1018 → TERM | "+" |
| | | | |
| GITELMAN | 7 BP DELETION | AA696 | |
| | CGT → CCT | R871 → P | |
| GITELMAN | CGT → CCT | R871 → P | |
| | | | |
| GITELMAN | GGT → GTT | Intron 24 - 5' splice | "+" |
| | | | |

| PHENOTYPE | MUTATION | CONSEQUENCE | HOMOZ. |
|---|---|---|---|
| GITELMAN | 4 bp Insertion | L998 → FS | "+" |
| | | | |
| GITELMAN | GAC → AAC | D486 → N | |
| | | | |
| GITELMAN | GTG → ATG | V647 → M | "+" |
| | | | |
| GITELMAN | TCG → TTG | S555 → L | |
| | CTT → CCT | L859 → P | |
| GITELMAN | GGT → GTT | Intron 9 - 5' splice | "+" |
| | | | |
| GITELMAN | CTT → CCT | L859 → P | |
| | ACC → AUC | T1026 → I | |
| GITELMAN | GGT → GTT | Intron 9 - 5' splice | |
| | GGG → AGG | G741 → R | |
| GITELMAN | CGC → CAC | R642 → H | |
| | CTC → CGC | L1010 → R | |
| GITELMAN | GCC → ACC | A464 → T | |
| | | | |
| GITELMAN | GGT → GTT | Intron 24 - 5' splice | |

Discussion

The finding of complete linkage of Gitelman's Syndrome with TSC, coupled with the finding of a large number of independent variants that are highly likely to disrupt TSC function and which are specific for Gitelman's Syndrome families, constitute proof that mutations in *TSC* cause Gitelman's Syndrome, the predominant subset of patients with Bartter's Syndrome. Whether the minority of patients with inherited hypokalaemic alkalosis and normal or hypercalciuria, so-called "true Bartter's" patients (Bettinelli, A., *et al.*, *J. Pediatr.* 120:38-43 (1992)), will prove to have mutations in this same gene or a different gene will require further study.

Disease pathogenesis

The diverse physiologic features of Gitelman's Syndrome must all stem from mutation in *TSC*. From the autosomal recessive inheritance and the known physiology of the syndrome, we propose that mutant alleles result in loss of normal TSC function leading to defective sodium and chloride reabsorption in the distal convoluted tubule. This defect would be expected to result in sodium plus chloride wasting, resulting in some degree hypovolemia and metabolic alkalosis. The reduced vascular volume activates the renin-angiotensin system, elevating renin and aldosterone levels. The elevated aldosterone levels lead to increased electrogenic sodium reabsorption via the epithelial sodium channel in an effort to defend intravascular volume. Sodium reabsorption via this channel is counterbalanced by potassium and hydrogen ion excretion, resulting in hypokalaemia and contributing to metabolic alkalosis. These are all characteristic features of Gitelman's Syndrome. How this primary defect in TSC function results in loss of magnesium and hypocalciuria remains a matter of speculation, however it is noteworthy that these same effects are seen in patients taking thiazide diuretics, specific inhibitors of the TSC; these observations further support the interpretation that *TSC* mutations result in loss of function. Importantly, it is now apparent that these latter abnormalities must be secondary consequences of primary defects in TSC.

Prevalence and transmission of Gitelman's alleles

Unusual segregation ratios and patterns of inheritance in some families have confused the understanding of the inheritance of Gitelman's Syndrome. The present linkage findings are consistent with genetic homogeneity of transmission as an autosomal recessive trait. The linkage findings support recessive inheritance more than 100,000-fold better than either dominant inheritance with incomplete penetrance or mitochondrial inheritance. The finding of affected subjects in different branches of some kindreds (e.g. GIT 102 and GIT 112) can now be explained in molecular terms by spouses in different branches introducing additional mutant alleles. For example, the 3 affected siblings of GIT102 (subjects 12, 14 and 15) are compound

heterozygotes for variants R421 and W209 (variants #3 and #4, Figure 1, Tables 1 and 4). R421 is also inherited by an affected second cousin, family member 11, by descent from their common ancestor; this subject is also a compound heterozygote, having inherited a third independent variant, altering a consensus splice site variant (variant #5; Tables 1 and 4, Figure 1) from her father.

Finding independent mutant alleles introduced into different branches of kindreds suggests that mutant alleles are not rare in the population. Moreover, the findings that consanguineous marriage is not prominent in Gitelman's Syndrome kindreds and that a high proportion of patients are compound heterozygotes are consistent with this notion. Also consistent with this notion is the remarkable conservation of 629 amino acids of this protein between humans and flounder, suggesting that many of these residues are required for normal protein function. These observations suggest that the potential target size for mutations generating Gitelman's Syndrome alleles will prove to be large, affording a relatively high rate of introduction of new mutant alleles into the population. The finding that thus far only one residue is mutated more than once on independent mutant alleles supports the contention of a large target size for Gitelman's mutations. While the true prevalence of this disease is unknown, minimum estimates of disease prevalence based on clinical features suggest a prevalence of heterozygotes of at least 1% in Swedish and Italian populations. Once the spectrum of mutations is defined, the true prevalence of mutant alleles in different populations can be determined independent of phenotypic effect.

Given the recessive inheritance of Gitelman's Syndrome, the high proportion of affected offspring of heterozygous parents in the Gitelman's kindreds studied to date is striking (Figure 1). After excluding index cases in these kindreds, 22/33 offspring of two heterozygous parents have Gitelman's Syndrome, far more than the expected 8 affected subjects. This high proportion of affected offspring could certainly result from ascertainment bias favoring identification of multiplex families with unusual segregation ratios. An alternative explanation would be segregation

distortion, with transmission of mutant alleles from heterozygous parents to more than the expected 50% of offspring. With the identification of mutant alleles, the segregation ratio of Gitelman's alleles in extended kindreds can be determined in order to directly assess this possibility without ascertainment bias.

Potential implications in heterozygotes

The identification of mutations that cause relatively severe disease in homozygotes raises the possibility that these alleles might have more modest effects in the much more common heterozygous state. One can imagine several possible phenotypes in Gitelman's heterozygotes, including modestly reduced blood pressure and/or predisposition to diuretic-induced hypokalaemia. Hypertension is a common multifactorial trait frequently associated with increased renal sodium reabsorption and sensitivity of blood pressure to the effects of dietary salt. Individuals heterozygous for Gitelman's mutations may be protected from development of hypertension by having modestly reduced renal sodium reabsorption, leading to a reduced set point of sodium balance, reduced intravascular volume and lower blood pressure. The potential impact of such alleles on blood pressure in the general population would be dependent on the true prevalence of such heterozygotes and their quantitative effect on blood pressure, both of which are unknown. Since the morbid clinical consequences of hypertension typically occur well after reproductive age, it seems unlikely that these alleles would increase reproductive fitness.

Hypokalaemic alkalosis is a relatively common complication of diuretic therapy of largely unknown cause. Mutant *TSC* alleles could contribute to development of this complication by further augmenting delivery of sodium and chloride to the distal nephron. This effect might be most likely in patients taking loop diuretics such as furosemide, and these mutations could consequently predispose to a particular complication of pharmacologic therapy.

Identification of specific mutations causing Gitelman's Syndrome permits testing of these hypotheses by identifying cohorts of heterozygous carriers and

comparing their blood pressures and responses to pharmacologic intervention to those of their homozygous wild-type siblings or other controls. In addition, subjects with unexplained hypokalaemia or hypokalaemia complicating drug therapy may be candidates for harboring Gitelman's mutations.

Finally, identification of the molecular basis of Gitelman's Syndrome provides for the genetic diagnosis of this disorder. At initial presentation, some patients with this disease are incorrectly believed to be diuretic abusers or to have bulimia. Indeed, one of the patients described herein was committed to a locked psychiatric ward and it was only when her hypokalaemia persisted for two weeks in this setting that a proper diagnosis was made. The ability to make a molecular genetic diagnosis is therefore of practical clinical benefit in many settings.

## EXAMPLE 2

Bartter's Syndrome, inherited hypokalaemic alkalosis with hypercalciuria, is caused by mutations in the Na-K-2Cl cotransporter NKCC2

### Methods

Bartter Syndrome Kindreds. BAR138 has been previously reported (Di Pietro, A. *et al., Ped. Med. Chir.* 13:279-280 (1991)). The other kindreds were recruited via ascertainment of a severely affected index case. Genomic DNA was prepared from venous blood of members of Bartter's Syndrome kindreds by standard procedures (Bell, G., Karam, J., *et al., Proc. Natl. Acad. Sci. (U.S.A.)* 78:5759-5763 (1981)).

Cloning and Characterization of the Human Genomic *NKCC2*. Oligonucleotide primers corresponding to either rabbit (Payne, J.A., *et al., Proc. Natl. Acad. Sci. (U.S.A.)* 91:4544-4548 (1994)) or rat (Gamba, G. *et al., J. Biol. Chem.* 26:17713-17722 (1994)) NKCC2 cDNA sequence were used to direct PCR using a human kidney cDNA library as template. The sequence of the corresponding human cDNA encoding NKCC2 was determined; sequences were determined from both

DNA strands. Segments from the 5' and 3' ends of the human *NKCC2* cDNA were radiolabeled and used to screen a human genomic PAC library35 by hybridization as described previously (Shimkets, R.A. *et al.*, *Cell* 79:407-414 (1994)). Intron-exon boundaries were determined by sequencing both strands of genomic DNA fragments. Genomic fragments bearing single introns and portions of adjacent exons of *NKCC2* were isolated by long-range PCR (Expand™ Long PCR System; Boehringer-Manheim) using PAC clone or genomic DNA as a template and primers likely to lie in within each exon based on the organization of the related gene *TSC*. DNA sequence analysis was by the dideoxy chain termination method using an ABI 373 instrument following a standard protocol.

Marker Development, Genotyping, and Linkage Analysis. Sau3AI subclones of the NKCC2 PAC clone were screened for (GT)n repeat sequences by hybridization as previously described (Shimkets, R.A. *et al.*, *Cell* 79:407-414 (1994)). This screen identified clone NKCGT7-3 which contained an array of (GT) (McCredie, D.A., *et al.*, *Aust. Paed. J.* 10:286-295 (1974)); primers flanking this simple sequence repeat were designed and used to direct PCR from genomic DNA of unrelated subjects (NKCGT7-3F: CACTAGGCTATTGTGTGGCTC; NKCGT7-3R: GTCTGTCCTCCACACTAG).

Genotyping of CEPH and disease kindreds for loci indicated in the text was performed by polymerase chain reaction using specific primers (Gyapay, G. *et al.*, *Nature Genet.* 7:246-339 (1994)) as described previously (Shimkets, R.A. *et al.*, *Cell* 79:407-414 (1994)), except that the products were labeled by inclusion of 1 μCi of [I-$^{32}$P]dCTP in the reaction mixture. All genotypes were scored independently by two investigators who were blinded to affection status. Analysis of linkage was performed using the LINKAGE programs Lathrop, G.M. *et al.*, *Proc. Natl. Acad. Sci. (U.S.A.)* 81:3443-3446 (1984)).

SSCP and DNA Sequencing. Molecular variants in genes were sought using single-strand conformational polymorphism (Orita, M. *et al.*, *Proc. Natl. Acad. Sci. (U.S.A.)* 86:2766-2770 (1989)). Primers for *TSC* were as described previously

(Example 1 and Simon, D.B. *et al.*, *Nature Genet.* 12:24-30 (1996)); exons of *NKCC2* were screened using 27 sets of specific primers (Table 6) based on the genomic organization of the gene. Primer pairs were employed to separately direct PCR using genomic DNA of disease family members or CEPH controls as a template as previously described (Shimkets, R.A. *et al.*, *Cell* 79:407-414 (1994)). Amplified products were analyzed for molecular variants by electrophoresis under 3 different non-denaturing conditions as well as under denaturing urea gels as previously described (Shimkets, R.A. *et al.*, *Cell* 79:407-414 (1994)). Identified variants were eluted from gel, reamplified by PCR, and DNA from both strands was sequenced in all cases. All SSCP genotypes were confirmed by independent amplifications.

Results

Bartter's Syndrome kindreds. 4 kindreds with at least one subject diagnosed with Bartter's Syndrome were studied. One of these cases has been reported previously (Di Pietro, A. *et al.*, *Ped. Med. Chir.* 13:279-280 (1991)). In three of these families, at least one affected subject is known to be the offspring of consanguineous union (Figure 6). In addition, in kindred BAR156 two second cousins of an index case are also affected. Index cases (Table 5) were all born prematurely with documented polyhydramnios and birth weight below 2 kg, and presented in the neonatal period with severe dehydration associated with marked hypokalaemia and hyperreninemic hyperaldosteronism. All had marked hypercalciuria and 3 had ultrasonographic evidence of nephrocalcinosis; none of these patients had hypomagnesemia. The early and severe presentation, hypercalciuria, polyhydramnios, and nephrocalcinosis all distinguish these patients from those previously classified as having Gitelman's Syndrome who were found to have mutations in TSC as the cause of their disease (Simon, D.B. *et al.*, *Nature Genet.* 12:24-30 (1996)).

Evaluation of *TSC*. To determine whether Bartter's Syndrome is due to mutations in the thiazide-sensitive Na-Cl cotransporter (TSC), we genotyped markers tightly linked to this locus on chromosome 16 in the consanguineous kindreds. If Bartter's Syndrome were due to mutations in TSC, we would anticipate that affected

subjects in these kindreds would be homozygous at the trait locus and at closely linked loci (Lander, E.S., *et al.*, *Science* 236:1567-1570 (1987)). D16S408 is linked to *TSC* at a recombination fraction of 1% (Simon, D.B. *et al.*, *Nature Genet.* 12:24-30 (1996)). This marker proved to be heterozygous in all of these affected subjects; analysis of additional closely linked flanking markers confirmed that none of these subjects have inherited two copies of the same haplotype (data not shown). These findings do not support mutation in TSC as the cause of Bartter's Syndrome. This conclusion is further supported by results of screening all 26 exons encoding the TSC protein for molecular variants by single strand conformational polymorphism (SSCP) (Orita, M. *et al.*, *Proc. Natl. Acad. Sci. (U.S.A.)* 86:2766-2770 (1989)). No variants altering the encoded protein were detected in any of these patients (data not shown).

Characterization of human *NKCC2*. In order to investigate NKCC2 for its potential role in Bartter's Syndrome, we characterized the human *NKCC2* locus (Figure 7). The predominant variant of human *NKCC2* cDNA isolated from a human kidney cDNA library encodes a protein of 1099 amino acids that shows strong sequence similarity to *NKCC2* cDNAs characterized from rabbit (Payne, J.A., *et al.*, *Proc. Natl. Acad. Sci. (U.S.A.)* 91:4544-4548 (1994)) (95% amino acid identity), and rat (Gamba, G. *et al.*, *J. Biol. Chem.* 26:17713-17722 (1994)) (93% identity). In addition, human NKCC2 shows considerable similarity in amino acid sequence to NKCC1 from shark rectal gland (Xu, J.-C. *et al.*, *Proc. Natl. Acad. Sci. (U.S.A.)* 91:2201-2205 (1994)) (60% identity) and the human TSC (Simon, D.B. *et al.*, *Nature Genet.* 12:24-30 (1996)) (47% identity).

Plasmid artificial chromosome (PAC) (Ioannou, P.A. *et al.*, *Nature Genet.* 6:84-89 (1994)) clones containing the human genomic NKCC2 locus were isolated, and one of these, NKCC2-6A, was found to contain all the coding exons. This permitted determination of the intron-exon structure of the gene (see Methods). The NKCC2 protein is encoded in 26 exons (Figure 7a ); introns range in length from 120 base pairs to 15 kb, and the coding region spans a total of 80 kb in genomic DNA. The location of intron-exon boundaries is very similar to those seen in TSC through

exon 19 (Simon, D.B. *et al.*, *Nature Genet.* 12:24-30 (1996)). Three alternative variants of exon 4 are encoded in genomic DNA, as reported in other species (Igarashi, P.*et al.*, *Am. J. Physiol.* 269:F405-F418 (1995)). By using primers from genomic sequences to direct PCR using DNA of somatic cell hybrids as a template, *NKCC2* was localized to human chromosome 15 (data not shown).

A (GT) dinucleotide repeat sequence was identified at the cloned *NKCC2* locus; this repeat proved to be polymorphic in genomic DNA, with 5 alleles and 42% heterozygosity in 50 unrelated subjects. This marker was genotyped in CEPH reference kindreds in order to localize *NKCC2* on the human genetic map; analysis revealed linkage to a cluster of loci at 15q15-21. Multipoint analysis yielded a maximum lod score of 13.3 for linkage to a 3 centimorgan interval flanked by D15S132 and D15S209 (Figure 7c); the odds favoring location in this interval were more than 100 times as likely as location in the next most likely interval (Figure 7c).

Homozygosity of *NKCC2* in Bartter's kindreds. We genotyped members of Bartter's Syndrome families for 4 markers in and flanking *NKCC2*. In each family, all affected offspring of consanguineous union were homozygous for alleles of each marker, and these haplotypes cosegregated with the disease, providing strong evidence supporting linkage of Bartter's Syndrome and the *NKCC2* locus (Figure 6). Moreover, while the affected subject in BAR138 is not known to be the product of consanguineous union, she too is homozygous for all markers tested in this segment.

Mutations in *NKCC2* in Bartter's Syndrome. These linkage data strongly suggest that Bartter's Syndrome is attributable to mutation at the *NKCC2* locus, motivating a search for functional mutations at this locus in these patients. Primers within introns of *NKCC2* were used to direct PCR from genomic DNA of affected subjects and the products were analyzed by SSCP. A single novel variant was found in each kindred and each cosegregated with the disease phenotype (Figures 6, 8). All these variants are homozygous in affected subjects with the exception of two affected siblings in BAR156. In this kindred, the index case is homozygous for a novel variant. Her affected second cousins have inherited one copy of the same variant

along with a portion of her haplotype; these subjects have presumably inherited a second, and as yet undetected, variant on the maternally inherited allele. None of these variants have been observed in examination of 80 alleles from unrelated subjects who do not have Bartter's Syndrome, and no variants altering the amino acid sequence have been detected in unrelated unaffected subjects.

Analysis of the DNA sequence of these variants reveals that each alters the encoded protein (Figure 8). The variant in BAR152 is a 1 base pair insertion introducing a frameshift mutation at codon M195 in the first transmembrane domain. The variant in BAR138 represents a 1 base pair deletion in codon R302 in the fourth transmembrane domain, also resulting in a frameshift mutation. Both of these mutations are extremely unlikely to yield proteins with normal function.

The other two variants result in non-conservative missense mutations at residues that are conserved among members of the NKCC family from distantly related species. The variant in BAR156 substitutes phenylalanine for valine at residue 272 in the third transmembrane domain. This valine residue is conserved in NKCC2 from human, rabbit, mouse, and rat, as well as NKCC1 in shark and human. Similarly, the variant in BAR165 substitutes an asparagine residue for aspartate at amino acid 648, just distal to the 12th transmembrane domain; this aspartate residue is also conserved in every member of the NKCC family.

**Table 5. Clinical characteristics of index cases with Bartter's Syndrome**

| Kindred | Location | $K^+$ | $HCO_3^-$ | UCa/UCr | Nephrocalcinosis | Preterm |
|---|---|---|---|---|---|---|
| BAR138 | Italy | 1.7 | 37 | 0.9 | No | Yes |
| BAR152 | Saudi Arabia | 3.1 | 27 | 0.7 | Yes | Yes |
| BAR156 | Saudi Arabia | 2.2 | 33 | 0.6 | Yes | Yes |
| BAR165 | Saudi Arabia | 1.9 | 27 | 0.5 | Yes | Yes |

$K^+$: serum potassium (mM, nl 3.5-5.0 mM); $HCO_3^-$: serum bicarbonate (mM, nl 23-26 mM); UCa/UCr: urinary calcium:creatinine ratio (mM:mM; nl ratio 0.2 - 0.4). Nephrocalcinosis determined by ultrasonography. Preterm: birth before 36 weeks' gestation. ND, not done.

Table 6. PCR Primers for SSCP Analysis of the Human NKCC2 Gene

| Primer Name | Exon | Forward Primer | Reverse Primer |
|---|---|---|---|
| hNKCC2ex1A | 1 | AACCACAAAGTAGATAGCTCAG | AACTGGCATCTGTTTTAGCAG |
| hNKCC2ex1B | 1 | GGGAATCAGGAGTGCTATGAC | AAGGGAGGAGACTTGCTTGTG |
| hNKCC2ex2 | 2 | TGTTCATTGACCAACTACTGTG | GCCTTGTTCACTCTTAATCCAC |
| hNKCC2ex3 | 3 | CACTATCGTTTGTCCTGTCTC | GTGACCTTCATCTCACATTCAG |
| hNKCC2ex4 | 4 | GATGTTTACCCTAGACTTGCTG | ACAAATGATGGTGCGGGTCAC |
| hNKCC2ex5 | 5 | CTAGCAGTTCCTCAATGTGAAG | ACTAAATTATGCTGCTTGGCAG |
| hNKCC2ex6 | 6 | ATGCTGCAATAAGACTCACATG | CAGGACCTGACCAGCCACTG |
| hNKCC2ex7 | 7 | GAGTCTTTCTGCAGTGGACAC | GAGGAGGGCAATGGAGAAG |
| hNKCC2ex8 | 8 | GTAACTTAATCTCCTGTACTGTG | TCCCAGGAATGCAAAGCAGAG |
| hNKCC2ex9 | 9 | CTCTGTATTCTTCTACCTCCAC | TGACATTCTGACACTGGAGAC |
| hNKCC2ex10 | 10 | TAGAAAACCGTAAGGGACCAG | TCAGAAATCTTACTGTATGTGAC |
| hNKCC2ex11 | 11 | ATGAAACAGATTCCAAATCACAG | AATAGGGAGAAGCACAAGCTG |
| hNKCC2ex12 | 12 | CTAGAGAAAATGACTGTGCATAG | GGAAAGCCCTATGAATAATCAG |
| hNKCC2ex13 | 13 | GCTCATCACTCATACGTACATG | CGTTTTATTGAGACAAACTAACTG |
| hNKCC2ex14 | 14 | GTGCCATGATCATAGTAGAGTG | TGGAAACGCTATTCCAGACAG |
| hNKCC2ex15 | 15 | TGCACAGAGGAAAGGTCAGTG | ACACCAGGATGCCTGAGACAC |
| hNKCC2ex16 | 16 | CAGGCTTCTTGCAGGGGCAC | GGAGGAAAAAGGACTTCCCTG |
| hNKCC2ex17 | 17 | CAGCAATGTGATATATAATAGCAG | GTGCTCATTCCCTCAATGCAG |
| hNKCC2ex18 | 18 | AGTACGGTAAGGATTGCCCAC | TATGTACTGCCCTGCTTAGTG |
| hNKCC2ex19 | 19 | GTAATACTAGTCCAAAGCTTGAG | TCAGGCACAAAGTAGGTGCTC |
| hNKCC2ex20 | 20 | GTAGTTCTGAGTTAAGTAGGTG | CATAGATGCTCAAATAGTGACTC |
| hNKCC2ex21 | 21 | GCCCTCAAAAGCAAACAGATG | CCCATATACCTTCTCATGCAG |
| hNKCC2ex22 | 22 | CCATTTAGATATACTCTTTGTGTC | TGAAATGACCTAACATGTGAGTG |
| hNKCC2ex23 | 23 | AAGCTAAGCTGAAATAAGACGTG | GTACCATGGGTAATCAATGTCTC |
| hNKCC2ex24 | 24 | GTTTCCCACTGTGAGGCCTC | CCTTTCTCAGCTAGTTAGACAG |
| hNKCC2ex25 | 25 | CATAATTCTGGTAGAACTGTACTC | TCCCACCTGAAGAGTCCCAAG |
| hNKCC2ex26 | 26 | CTAGTGCCGTTACTACCTATAG | GATCAGATTTACAACATAAAGTAC |

Primers are all within introns with the exception of: hNKCC2ex1A-reverse and hNKCC2ex1B-forward, which lie within exon 1; hNKCC2ex26-reverse lies distal to the normal termination codon.

Table 7. Summary of mutations in *NKCC2* that cause Bartter's Syndrome

| Phenotype | Mutation | Consequence | Homozygous |
|---|---|---|---|
| Bartter | ATG → AATG | M195 → Frameshift | + |
| Bartter | CGG → GG | R302 → Frameshift | + |
| Bartter | GAC →AAC | D648 → N | + |
| Bartter | GTT → TTT | V272 → F | + |
| Bartter | CAG → CTAG | Q823 → Frameshift | + |
| Bartter | TCC → CCC | S507 → P | |

Discussion

Identification of independent mutations in *NKCC2* that segregate with the disease, show specificity for Bartter's Syndrome kindreds, and result in frameshifts or non-conservative amino acid substitutions in highly conserved residues, constitutes proof that mutations in the renal absorptive Na-K-2Cl cotransporter cause Bartter's Syndrome. This finding establishes a molecular basis for this disease and permits genetic distinction of this disorder from Gitelman's Syndrome. These findings expand knowledge of the molecular mechanisms underlying inherited diseases of renal ion transport. Mutations in 5 different genes mediating renal sodium reabsorption are now implicated in 4 different mendelian disorders (Figure 9) (Shimkets, R.A. *et al.*, *Cell* 79:407-414 (1994); Hansson, J.H. *et al.*, *Nature Genet.* 11:76-82 (1995); Chang, S.S. *et al.*, *Nature Genet.* 12:248-253 (1996); Simon, D.B. *et al.*, *Nature Genet.* 12:24-30 (1996)). In addition, mutations leading to increased activity of the mineralocorticoid receptor, including glucocorticoid-remediable aldosteronism (Lifton, R.P. *et al.*, *Nature* 355:262-265 (1992)) and the syndrome of apparent mineralocorticoid excess (Mune, T. *et al.*, *Nature Genet.* 10:394-399 (1995)), act by increasing activity of the epithelial sodium channel of the distal nephron.

Disease pathophysiology. Virtually all features of the pathophysiology of Bartter's Syndrome can be explained by mutations in the Na-K-2Cl cotransporter leading to loss of function. This cotransporter is located in the thick ascending limb of the loop of Henle and accounts for reabsorption of approximately 30% of the filtered load of sodium (Greger, R., *Physiol. Rev.* 65:760-797 (1985)) (Figure 9); consequently, loss of function leads to marked salt wasting and volume contraction, accounting for the dehydration seen in affected patients. This results in secretion of renin and elevated aldosterone levels, producing increased electrogenic sodium reabsorption via the amiloride-sensitive epithelial sodium channel of the distal nephron. Sodium reabsorption via this channel is indirectly coupled to potassium and hydrogen ion secretion, producing the characteristic hypokalaemic alkalosis.

The reabsorption of approximately 25% of filtered calcium also occurs in the thick ascending limb (Sutton, R.A.R., *et al.*, In The Kidney (eds. Brenner, B.M. & Rector, F.C.) 551-618 (Saunders, Philadelphia, 1981)) and this is coupled to sodium reabsorption in this nephron segment. Loss of sodium reabsorption here would consequently be expected to result in the characteristic hypercalciuria and nephrocalcinosis seen in these patients.

Urinary prostaglandin E2 levels have been reported to be elevated in patients with Bartter's Syndrome (Seyberth, H.W. *et al.*, *Pediatr. Nephrol.* 1:491-497 (1987); Gill, J.R., Jr., *et al.*, *Am. J. Med.* 61:43-51 (1976)); high PGE2 levels can be produced by administration of loop diuretics (Dunn, M.J., *Kidney Int.* 18:86-102 (1981)), consistent with genetic loss of Na-K-2Cl function resulting in a secondary increase in PGE2 levels.

Classification of hypokalaemic alkalosis. It is now apparent that inherited hypokalaemic alkalosis is genetically heterogeneous and due to mutations in genes encoding at least two proteins -- the Na-K-2Cl cotransporter of the thick ascending limb of the loop of Henle (NKCC2) and the Na-Cl cotransporter of the distal convoluted tubule (TSC). Thus far, the clinical and physiologic features of patients with mutations in these two genes are readily distinguishable -- all patients ascertained with hypokalaemic alkalosis plus neonatal presentation and hypercalciuria have mutations in NKCC2, while all patients ascertained with hypokalaemic alkalosis plus presentation after age 8 and hypocalciuria have had mutations in TSC (Simon, D.B. *et al.*, *Nature Genet.* 12:24-30 (1996)).

Whether all inherited hypokalaemic alkalosis with normal or low blood pressure will prove due to mutation in one of these two genes or whether further genetic heterogeneity will be found is unresolved. A group of patients has been described with severe magnesium wasting, hypokalaemic alkalosis and nephrocalcinosis (Gullner Syndrome) (Gullner, H.-G., *et al.*, *Am. J. Med.* 71:578-582 (1981)). The hypokalaemia in these patients has been reported to be corrected with magnesium replacement. These patients seem likely to be another distinct subset of

the spectrum of inherited hypokalaemic alkalosis. The finding of high urinary prostaglandin levels in some patients with, typically, neonatal hypokalaemic alkalosis, has led to suspicion that hyperprostaglandiuria might represent a distinct syndrome (hyperprostaglandinuria E syndrome) (Seyberth, H.W. *et al.*, *Pediatr. Nephrol.* 1:491-497 (1987)), however the present results suggest that this group of patients will likely prove to have mutations in NKCC2 with hyperprostaglandinuria as a secondary phenomenon. At present, the evidence for two distinct clinical syndromes supported by mutations in two different genes supports a logical framework for classification of these patients as having either Gitelman's Syndrome or Bartter's Syndrome.

Conversely, the spectrum of clinical and physiologic features resulting from mutations in these two genes also remains to be defined. Patients who have been studied thus far represent extremes of the phenotypic spectra. Some patients with hypokalaemic alkalosis have been reported to have normal urinary calcium; these patients could have mutations in *NKCC2*, TSC or another gene. Further examination of genotype-phenotype relationships and specific phenotypic consequences of particular mutant alleles will consequently be of interest, and should permit rigorous classification based on analysis of mutations and clinical features.

Disease prevalence. The proteins encoded by *NKCC2* and *TSC* are closely related, with similar highly conserved sequence, structure and function. From this, one might expect the prevalence of mutant alleles in these genes, and the prevalence of clinical disease, to be similar. However, from our experience in recruiting published patients with hypokalaemic alkalosis, it appears that patients with Gitelman's Syndrome (defined by the presence of hypocalciuria) grossly outnumber those with Bartter's Syndrome (defined by the presence of hypercalciuria) (Simon *et al.*, unpublished data). The greater morbidity and mortality associated with Bartter's Syndrome could reduce reproductive fitness, resulting in increased loss of mutant *NKCC2* alleles from the population.

There may also be a difference in the rate of introduction of new mutant alleles at these loci. The most common site for single base substitution in mammals is at

CpG dinucleotides owing to methylation at these cytosine residues and subsequent deamination to thymidine (Cooper, D.N., *et al.*, *Hum. Genet.* 83:181-188 (1989)). There is a striking difference in the prevalence of CpG dinucleotides in coding regions of these two genes. Cytosine residues of CpG dinucleotides occupy the first or second codon positions of 60 codons in *TSC* but only 30 such sites in *NKCC2*. This difference is not merely due to differences in amino acid sequence, but is reflected in significant differences in codon usage. For example, while 30 of 48 arginine codons in *TSC* have CpG dinucleotides, only 15 of 43 arginine residues in *NKCC2* utilize such codons ($c2,1\ df = 6.99$, $p < 0.01$). These differences may reflect stronger selection against CpG dinucleotides in *NKCC2*, and may in part account for the large difference in prevalence G-C base pairs in coding regions of these two genes (59% in *TSC*, 45% in *NKCC2*).

Potential implications in heterozygotes. While mutant *NKCC2* alleles have severe effects when homozygous, they may also have detectable phenotypes in the more prevalent heterozygotes. Among potential phenotypes, lowered blood pressure is a reasonable possibility, owing to reduced sodium reabsorption in the thick ascending limb, leading to a reduced set point of sodium balance and decreased intravascular volume. The ability of loop diuretics to lower blood pressure by inhibition of *NKCC2* supports this possibility.

Another potential phenotype in heterozygotes is susceptibility to diuretic-induced hypokalaemia. Thiazide diuretics are commonly used anti-hypertensive agents that inhibit TSC in the distal convoluted tubule; thiazide-induced hypokalaemia is a relatively common complication. Heterozygotes for NKCC2 mutations may be able to compensate for diminished sodium reabsorption in the thick ascending limb by increased distal reabsorption via TSC in the distal convoluted tubule (Ellison, D.H. *Ann. Int. Med.* 114:886-894 (1991)) (Figure 9). Inhibition of this latter cotransporter by thiazides could, however, result in marked salt wasting which could only be compensated by increased activity of the epithelial sodium channel in the distal nephron at the expense of marked potassium loss. Heterozygous

NKCC2 mutations could consequently predispose to this specific complication of pharmacologic therapy, and such heterozygous carriers could constitute a significant fraction of patients developing this complication.

Homozygotes for NKCC2 mutations have marked urinary calcium wasting, leading to both early and severe nephrocalcinosis and marked demineralization of bone. Salt wasting in heterozygotes for NKCC2 mutations might also result in hypercalciuria, potentially increasing susceptibility to nephrolithiasis (renal stones) and osteoporosis (bone demineralization), multifactorial traits with known inherited components. Interestingly, mutations in an X-linked renal chloride channel have been shown to promote development of nephrolithiasis (Lloyd, S.E. *et al.*, *Nature* 379:445-449 (1996)), consistent with the notion that primary abnormalities in renal sodium and chloride handling may underlie stone development in a significant fraction of affected subjects.

In addition to these mutations believed to cause loss of transport function, it is possible that variants in this gene could also lead to gain of function, and potentially contribute to development of hypertension. A comparable situation has been demonstrated for the amiloride-sensitive epithelial sodium channel in which gain-of-function mutations cause the hypertensive disorder Liddle Syndrome (Shimkets, R.A. *et al.*, *Cell* 79:407-414 (1994); Hansson, J.H. *et al.*, *Nature Genet.* 11:76-82 (1995)) while loss of function mutations cause the salt wasting disease pseudohypoaldosteronism type I (Chang, S.S. *et al.*, *Nature Genet.* 12:248-253 (1996)) (Figure 9).

## EXAMPLE 3

Genetic heterogeneity of Bartter's Syndrome, hypokalaemic alkalosis with hypercalciuria, revealed by mutations in the $K^+$ channel ROMK

Methods

Bartter's Syndrome kindreds. Kindreds were recruited via ascertainment of affected index cases. Genomic DNA was prepared from venous blood of members of Bartter's Syndrome kindreds by standard procedures (Bell, G., *et al.*, *Proc. Natl. Acad. Sci. (U.S.A.)* 78:5759-5763 (1981)).

Characterization of human genomic ROMK intron-exon junctions. Genomic fragments bearing single introns and portions of adjacent exons of ROMK were isolated by long-range PCR (ExpandTM Long PCR System; Boehringer-Mannheim) using genomic DNA as a template and primers within each exon based on published data (Shuck, M.E. *et al.*, *J. Biol. Chem.* 269:24261-24270 (1994)). DNA sequence analysis of products was by the dideoxy chain termination method using an ABI 373A instrument following a standard protocol. Intron sequences were used to define primers amplifying intron-exon boundaries and coding regions of ROMK (Table 9); additional junction sequences have been submitted to GENBANK.

Genotyping and linkage analysis. Genotyping of loci indicated in the text was performed by polymerase chain reaction using specific primers as described previously (Simon, D.B., *et al.*, *Nature Genet.* 13:183-188 (1996)). All genotypes were scored independently by two investigators who were blinded to affection status. Analysis of linkage was performed using the LINKAGE programs (Lathrop, G.M. *et al.*, *Proc. Natl. Acad. Sci. (U.S.A.)* 81:3443-3446 (1984)), specifying Bartter's Syndrome as an autosomal recessive trait with 99% penetrance and a prevalence of phenocopies of 0.1%.

SSCP and DNA sequencing. Molecular variants in genes were sought using single-strand conformational polymorphism (Orita, M. *et al.*, *Proc. Natl. Acad. Sci.*

(U.S.A.) 86:2766-2770 (1989)). Primers for *NKCC2* and *TSC* were as described previously (Examples 1 and 2 and Simon, D.B. *et al.*, *Nature Genet.* 12:24-30 (1996), Simon, D.B., *et al.*, *Nature Genet.* 13:183-188 (1996)). Primer pairs were employed to direct PCR using genomic DNA of disease family members or controls as a template as previously described (Simon, D.B. *et al.*, *Nature Genet.* 12:24-30 (1996)). All primer pairs generate products with sizes between 150 and 300 base pairs. Amplified products were analyzed for molecular variants by electrophoresis under 2 different non-denaturing conditions as well as on denaturing urea gels as previously described (Simon, D.B. *et al.*, *Nature Genet.* 12:24-30 (1996)). Identified variants were eluted from gel, reamplified by PCR, and DNA from both strands was sequenced in all cases. All SSCP variants were confirmed by independent amplifications.

Results

Bartter's Syndrome Kindreds

We investigated subjects from 9 previously unreported kindreds with Bartter's Syndrome, defined by the presence of hypokalaemic metabolic alkalosis with salt wasting and low blood pressure in association with hypercalciuria and presentation in the neonatal period (Table 8). All patients had markedly elevated plasma aldosterone levels and plasma renin activities (data not shown). The clinical and biochemical features of these patients are indistinguishable from those found in patients previously found to have mutations in NKCC2, and are distinct from those found in patients with mutations in TSC.

Analysis of NKCC2

Affected subjects in 6 of these kindreds were known to be offspring of marriage between first cousins (Table 8). In these kindreds, it is highly likely that affected subjects are homozygous for identical disease mutations as well as flanking markers by descent from a heterozygous great-grandparent. Testing for homozygosity of intragenic and flanking markers is consequently a test of linkage in such kindreds

(Lander, E.S. *et al.*, *Science* 236:1567-1570 (1987)). Accordingly, we genotyped highly polymorphic markers spanning a 3 cM interval containing *NKCC2* in these consanguineous kindreds wre genotyped; these loci have proved homozygous in 5 previously studied kindreds with Bartter's Syndrome due to mutation in *NKCC2*. In 3 of these consanguineous kindreds (BAR157, BAR181, BAR182), all affected subjects are homozygous for *NKCC2* haplotypes, consistent with Bartter's Syndrome in some or all of these kindreds being attributable to mutation in *NKCC2*, as reported previously (Example 2 and Simon, D.B., *et al.*, *Nature Genet.* 13:183-188 (1996)) (data not shown). In contrast, in kindreds BAR159 and BAR161, homozygosity by descent at *NKCC2* is excluded (Figure 10). Moreover, in BAR159, two affected siblings are discordant for *NKCC2* haplotypes, and an unaffected sib shares *NKCC2* haplotypes with an affected sib; traditional analysis of linkage in this kindred under a conservative model of the trait locus (see Methods) rejects linkage with a lod score of -3.4. In addition, affected members of neither kindred are homozygous for loci tightly linked to *TSC* (data not shown). These findings provide strong evidence of genetic heterogeneity of Bartter's Syndrome, and indicate that mutation in *NKCC2* does not cause the disease in BAR159 and BAR161. This conclusion is supported by the failure to identify mutations in *NKCC2* in affected subjects of these families by screening all 26 exons encoding NKCC2 (Simon, D.B., *et al.*, *Nature Genet.* 13:183-188 (1996)) by single-strand conformational polymorphism (SSCP) (Orita, M. *et al.*, *Proc. Natl. Acad. Sci.* (U.S.A.) 86:2766- 2770 (1989)) (data not shown).

Analysis of ROMK

These observations motivated consideration that loss of function mutations in a proposed regulator of NKCC2 function, ROMK, could account for the disease in some of these families. The gene encoding ROMK has been cloned, and intron-exon organization defined (Yano, H., *et al.*, *Mol. Pharmacology* 45:854-860 (1994); Shuck, M.E. *et al.*, *J. Biol. Chem.* 269:24261-24270 (1994)): there are 5 exons which are used in varying combinations to produce 3 distinct ROMK proteins that differ at the amino terminus and vary in length from 372 to 391 amino acids in humans (Shuck,

M.E. *et al.*, *J. Biol. Chem.* 269:24261-24270 (1994)). All isoforms share a core of 372 amino acids encoded by exon 5; this 372 amino acid protein corresponds to isoform ROMK2. The published intron sequence data was supplemented with additional intron sequence information disclosed herein to permit examination of all 5 exons and intron-exon boundaries (see Methods).

Affected subjects of BAR159 and BAR161 were screened for ROMK mutations by SSCP. Homozygous ROMK variants were found in affected subjects of both kindreds, and these variants cosegregated with the disease (Figures 10 and 11a, b). These variants are not common in the population, as they are not detected on 80 chromosomes from unaffected unrelated subjects from Saudi Arabia and the CEPH reference pedigrees. By homozygosity mapping, specifying these variants as rare, the lod score for linkage of ROMK and Bartter's Syndrome in these two families is 3.2, supporting linkage. Analysis of the DNA sequence of these variants demonstrates that the homozygous variant in BAR159 (Figure 11a) changes codon 60 (codon number in *ROMK2*) from TAC, encoding tyrosine, to TAG, specifying chain termination and truncating the encoded protein prior to the first transmembrane domain (Figure 12). The homozygous variant in BAR161 results in insertion of a single T-A base pair into a sequence of 6 consecutive T residues spanning codons 13 and 14 (Figure 11b), resulting in a frameshift mutation changing the encoded protein from amino acid 15 onward (Figure 12), resulting in premature termination at codon 54. These findings provide strong evidence that mutations in *ROMK* cause Bartter's Syndrome in these two families.

The 7 additional Bartter's kindreds were then screened for *ROMK* variants. Two variants were found in each of 2 outbred kindreds that had revealed no variants in screening of *NKCC2*; none of these variants were found in 80 chromosomes from unaffected subjects. In BAR208, one variant results in a missense mutation, substituting arginine for serine at codon 200 (Figures 11d, 12). This serine is highly conserved among members of the IRK family of $K^+$ channels and represents a protein kinase A phosphorylation site in the cytoplasmic carboxy terminus of the protein; this

site has been shown to be phosphorylated by PKA, and substitution of alanine by site-directed mutagenesis has shown that this site is required for normal $K^+$ channel activity, with mutant ROMK showing an approximately 50% reduction in channel activity (Xu, Z-C., *et al.*, *J. Biol. Chem.* 271:9313-9319 (1996)). The second variant in this kindred, present on the other *ROMK* allele, represents another premature termination codon, occurring at codon 58, again truncating the encoded protein prior to the first transmembrane domain (Figure 12). In BAR206, one variant represents a 4 base pair deletion, spanning the last base of codon T313 and all of codon K314, resulting in a frameshift mutation and altering the encoded protein from amino acid 315 onward, ending at a new stop codon at position 350 (Figures 11c, 12). The second variant in this kindred arises from substitution of valine for alanine at amino acid 195, in the cytoplasmic carboxy terminus of ROMK (Figure 12); this alanine residue is conserved in rat and human ROMK, and has not been observed in unrelated unaffected subjects. In one additional outbred kindred, BAR139 in which no NKCC2 variants have been identified, a single ROMK variant, substituting threonine for methionine at amino acid M338 was identified (Figure 12); this variant has not been seen on 80 chromosomes from unaffected subjects. As yet, no mutation has been identified on the other *ROMK* allele in this affected subject.

**Table 8. Clinical characteristics of index cases with Bartter's Syndrome**

| Kindred | Ancestry | $K^+$ | $HCO_3^-$ | UCa/UCr | Nephrocalcinosis | Consang. |
|---|---|---|---|---|---|---|
| BAR159 | Saudi Arabia | 3.2 | 27 | 0.5 | Yes | Yes |
| BAR161 | Saudi Arabia | 2.7 | 33 | 1.1 | Yes | Yes |
| BAR206 | Spain | 3.3 | 32 | 1.0 | Yes | No |
| BAR208 | Spain | 3.4 | 28 | 0.9 | Yes | No |
| BAR139 | Italy | 1.8 | 36 | 0.9 | No | No |
| BAR144 | Pakistan | 2.5 | 31 | 0.8 | Yes | No |
| BAR157 | Yemen | 1.1 | 52 | 1.0 | Unknown | Yes |
| BAR181 | Saudi Arabia | 2.2 | 31 | 1.1 | Yes | Yes |
| BAR182 | Saudi Arabia | 1.9 | 35 | 0.8 | Yes | Yes |

$K^+$: serum potassium (mM, nl 3.5-5.0 mM); $HCO_3^-$: serum bicarbonate (mM, nl 23-26 mM); UCa/UCr: urinary calcium:creatinine ratio (mM:mM, nl <0.4 ). Nephrocalcinosis determined by ultrasonography. Consang.: Offspring of consanguineous union.

Table 9. PCR Primers for SSCP Analysis of the Human ROMK Gene

| Primer Name | Exon | Forward Primer | Reverse Primer |
|---|---|---|---|
| hROMKex1 | 1 | TGCCATACAGATGAGTTGGCAG | CCTACAAAGAGACAATGAGGTG |
| hROMKex2 | 2 | AAAGGCAAGAGTTAGCCAGAC | CTGTAAGATACAGATATTGGGAG |
| hROMKex3 | 3 | CTCCAGACAGTAGCCATATGTG | TTTCTTGTAGCCTGGGGTGTC |
| hROMKex4 | 4 | AGCGTCAGTCCACTGACTGTC | GCCTGGCTTTCCAGAGAGGTG |
| hROMKex5A | 5 | CATGTGGGTCACCTAGTTCAC | TACCGTTGTCCAGATGTCCAC |
| hROMKex5B | 5 | TGGCAATGTGGAGGCACAGTC | TGGTCACTTGAGTCTCCAGAG |
| hROMKex5C | 5 | AATCACACTCCCTGTGTGGAG | CTGAACGTAATGGTCTTGGCAC |
| hROMKex5D | 5 | CATCTTAGCCAAGATCTCCAG | CTACAAAGTTGATATTGATCTGGTC |
| hROMKex5E | 5 | AGTCACTCCTGAAGGAGAGAC | GCACTGGTGGACTCCACTGTG |
| hROMKex5F | 5 | ATGGCAGCGGAGACCCTTCTC | GGGGTCTCCACTTCCACTGTC |
| hROMKex5G | 5 | AAAGGAAGGGAAATACCGAGTG | AGGTACTAGGAGCTTTAGAGAC |

Primers hROMKex1, hROMKex2, hROMKex3, hROMKex4, and hROMKex5A-forward are within introns. All other primers lie within exon 5.8

Table 10. Summary of mutations in *ROMK* that cause Bartter's Syndrome

| Phenotype | Mutation | Consequence | Homozygous |
|---|---|---|---|
| Bartter | TAC → TAG | Y60 → TERM | + |
| Bartter | TTT → TTTT | F13 → Frameshift | + |
| Bartter | AGC → AGG | S200 → R | |
| Bartter | TGG → TAG | W58 → TERM | |
| Bartter | CAAAGG → CG | T313 → Frameshift | |
| Bartter | GCT → GTT | A195 → V | |
| Bartter | ATG → ACG | M338 → T | |

Discussion

The finding of independent *ROMK* mutations that drastically alter channel structure and that cosegregate with Bartter's Syndrome demonstrates that mutations in *ROMK* cause Bartter's Syndrome. These findings establish genetic heterogeneity of Bartter's Syndrome and have implications for genetic testing for this disease. Moreover, they strongly support the role of ROMK in the regulation of renal Na-K-2Cl cotransport activity and net salt reabsorption *in vivo* by recycling $K^+$ entering cells of the TAL back to the lumen. Whether mutations in *NKCC2* and *ROMK* are the sole genes in which mutation causes Bartter's Syndrome, or whether additional genes will be identified remains an open question; of the total of 15 families harboring independent Bartter's mutations studied thus far (Simon, D.B., *et al.*, *Nature Genet.*

13:183-188 (1996) and the present study), *NKCC2* variants have been identified or implicated by linkage in 10 families, and *ROMK* mutations have been identified in 5 families. In one outbred family mutation in either ROMK or NKCC2 have not been found to date. This could reflect either incomplete sensitivity of mutation detection or alternatively could be accounted for by further genetic heterogeneity. The present findings bring to 6 the number of renal ion channels, subunits or transporters in which mutation has been shown to affect blood pressure in humans (Lifton R.P., *Science* 272:676-680 (1996); Simon, D.B., *et al.*, *Nature Genet.* 13:183-188 (1996)).

The finding of homozygous premature termination and frameshift mutations early in the encoded protein (Figure 12) provides very strong evidence that these ROMK mutations result in loss of potassium channel activity; this suggestion is further supported by finding mutation in a PKA phosphorylation site that has been shown by expression in Xenopus oocytes to be required for full ROMK activity (Xu, Z-C., *et al.*, *J. Biol. Chem.* 271:9313-9319 (1996)). In addition, a distal frameshift mutation deleting the last 58 normal amino acids strongly suggests an essential role of this carboxyl terminus in normal potassium channel function. The functional consequences of this mutation and two additional missense variants, A195V and M338T, can be assessed by expression studies; these may provide new insight into channel structure and function. It should be noted that all the mutations identified to date are in the core peptide shared by all known ROMK isoforms, and consequently activity of all isoforms is expected to be affected by these mutations.

The pathophysiology seen in affected patients can be explained by the loss of ROMK function resulting in inability to recycle $K^+$ from cells of the TAL back into the renal tubule, resulting in $K^+$ levels in the lumen that are too low to permit continued Na-K-2Cl cotransport activity, producing salt wasting from this nephron segment. This salt wasting results in activation of the renin-angiotensin system, increased aldosterone levels, and increased electrogenic sodium reabsorption via the epithelial sodium channel of the distal nephron in exchange for $K^+$ and $H^+$, accounting for the observed hypokalaemic alkalosis. Since calcium reabsorption is coupled to

$Na^+$ reabsorption in the TAL, loss of $Na^+$ transport in this segment leads to the characteristic hypercalciuria of these patients.

While at least one additional secretory potassium channel has been identified in the TAL5, the present results indicate that these two $K^+$ channels are not redundant in function, as this other channel cannot substitute for loss of ROMK function. ROMK isoforms are also expressed at more distal sites in the nephron, and it has been proposed that isoforms of this same channel contribute to net renal potassium secretion in the distal nephron (Lee, W.S., *et al.*, *Am. J. Physiol.* (Renal Fluid Electrol. Physiol.) 268:F1124-31 (1995), Boim, M.A. *et al.*, *Am. J. Physiol.* (Renal Fluid Electrol. Physiol.) 268:F1132-40 (1995), Hebert, S.C., *Kidney Int.* 48:1010-1016 (1995)). Since distal secretion is the major determinant of net renal potassium secretion, loss of this secretory potassium channel would be expected to result in impaired potassium secretion in response to aldosterone. As a consequence, one might expect to be able to distinguish Bartter's patients with NKCC2 mutations (and unimpaired distal $K^+$ secretion) from those with mutations in ROMK by higher potassium levels in the latter patients. In retrospect, the 4 patients with ROMK mutations identified on both alleles show a trend toward higher $K^+$ levels (Table 2 and Simon, D.B., *et al.*, *Nature Genet.* 13:183-188 (1996)), however they all still are well below the normal range. This finding suggests that ROMK isoforms do not play an indispensable role in distal renal potassium excretion in vivo in humans. Further work will be required to address this question and to determine whether the clinical features of patients with mutations in these two genes can be distinguished clinically. In addition, while ROMK isoforms are also expressed in brain, spleen, lung and eye (Hebert, S.C., *Kidney Int.* 48:1010-1016 (1995)), there are no obvious phenotypes in these patients that suggest an essential role for ROMK function in these organs.

The finding that homozygous loss of ROMK function alters renal sodium handling raises the possibility that heterozygous carriers of ROMK mutations might have less drastic phenotypes owing to haploinsufficiency. These potential phenotypes would be expected to be similar to those previously suggested for NKCC2 mutant

heterozygotes (Simon, D.B., *et al.*, *Nature Genet.* 13:183-188 (1996)), including reduced blood pressure, and predisposition to osteoporosis and/or nephrolithiasis. With the ability to identify heterozygous carriers, these possibilities can be tested. Similarly, the finding that increased renal sodium reabsorption underlies many variants of hypertension raises the question of whether gain of function mutations in ROMK could result in increased renal sodium reabsorption and contribute to elevated blood pressure. Identification of ROMK as an important regulator of net renal sodium reabsorption motivates determination of whether variants in this gene as well as in regulators of ROMK activity play a role in the determination of blood pressure in humans. A skilled artisan can readily practice the inventions disclosed herein following the methods and Examples provided herein.

Claims

1. A method to determine the presence or absence of a mutation conferring a pathological condition mediated by altered ion transport, said method comprising the step of analyzing a nucleic acid sample for the presence of a mutation in a human gene selected from the group consisting of the human thiazide-sensitive Na-Cl cotransporter gene, *TSC*; the human ATP-sensitive $K^+$ channel gene, *ROMK*; and the human Na-K-2Cl cotransporter gene, *NKCC2*.

2. The method of claim 1 wherein said method is used to determine the presence or absence of a nucleic acid molecule that is characteristic of a pathological condition selected from the group consisting of Bartter's Syndrome, Gitelman's Syndrome, hypokalaemic alkalosis, hypokalaemic alkalosis with hypercalciuria, kidney stones, high blood pressure, osteoporosis and sensitivity to diuretic-induced hyperkalaemia.

3. The method of claim 1 wherein said nucleic acid sample is analyzed for the presence of a nucleic acid molecule encoding a human TSC protein that is altered for one or more amino acids when compared to a wild-type human TSC protein, wherein alterations in the TSC protein are diagnostic of Gitelman's Syndrome (homozygous alteration) or a carrier of Gitelman's Syndrome (heterozygous alteration).

4. The method of claims 5, wherein said nucleic acid sample is analyzed for the presence of a nucleic acid molecule encoding a human TSC protein whose amino acid sequence is altered for one or more amino acids from an amino acid sequence selected from the group consisting of wild-type TSC sequences.

5. The method of claim 6 wherein said nucleic acid sample is analyzed for the presence of a nucleic acid molecule encoding a human TSC protein with an amino acid sequence selected from the group consisting of altered TSC sequences.

6. The method of claim 1 wherein said nucleic acid sample is analyzed for the presence of a nucleic acid molecule encoding a human ROMK protein that is altered for one or more amino acids when compared to a wild-type human ROMK protein, wherein alterations in the ROMK protein are diagnostic of Bartter's Syndrome (homozygous state) or a carrier of Bartter's Syndrome (heterozygous state).

7. The method of claims 6, wherein said nucleic acid sample is analyzed for the presence of a nucleic acid molecule encoding a human ROMK protein whose amino acid sequence is altered for one or more amino acids from an amino acid sequence selected from the group consisting of wild-type ROMK sequences.

8. The method of claim 7 wherein said nucleic acid sample is analyzed for the presence of a nucleic acid molecule encoding a human ROMK protein with an amino acid sequence selected from the group consisting of altered ROMK sequences.

9. The method of claim 1 wherein said nucleic acid sample is analyzed for the presence of a nucleic acid molecule encoding a human NKCC2 protein that is altered for one or more amino acids when compared to a wild-type human NKCC2 protein, wherein alterations in the NKCC2 protein are diagnostic of Bartter's Syndrome (homozygous state) or a carrier of Bartter's Syndrome (heterozygous state).

10. The method of claims 9, wherein said nucleic acid sample is analyzed for the presence of a nucleic acid molecule encoding a human NKCC2 protein whose amino acid sequence is altered for one or more amino acids from an amino acid sequence selected from the group consisting of wild-type NKCC2 sequences.

11. The method of claim 10 wherein said nucleic acid sample is analyzed for the presence of a nucleic acid molecule encoding a human NKCC2 protein with an amino acid sequence selected from the group consisting of mutant NKCC2 sequences.

12. The method of claim 3 wherein said method comprises the steps of amplifying nucleic acid molecules in said sample using a nucleic acid amplification method and primers that flank and selectively amplify at least one exon of said TSC encoding nucleic acid molecule and identifying whether a mutation is present in said amplified nucleic acid molecule.

13. The method of claim 6 wherein said method comprises the steps of amplifying nucleic acid molecules in said sample using a nucleic acid amplification method and primers that flank and selectively amplify at least one exon of said ROMK1 encoding nucleic acid molecule and identifying whether a mutation is present in said amplified nucleic acid molecule.

14. The method of claim 9 wherein said method comprises the steps of amplifying nucleic acid molecules in said sample using a nucleic acid amplification method and primers that flank and selectively amplify at least one exon of said NKCC2 encoding nucleic acid molecule and identifying whether a mutation is present in said amplified nucleic acid molecule.

15. A method to determine the presence or absence of a mutated protein conferring altered ion transport, said method comprising the step of analyzing a protein sample for the presence of a mutation in a protein selected from the group consisting of the human thiazide-sensitive Na-Cl cotransporter protein, TSC; the human ATP-sensitive $K^+$ channel protein, ROMK; and the human Na-K-2Cl cotransporter protein, NKCC2.

16. The method of claim 15 wherein said method is used to determine the presence or absence of a mutated protein that is characteristic of a pathological condition selected from the group consisting of Bartter's Syndrome, Gitelman's Syndrome, hypokalaemic alkalosis, hypokalaemic alkalosis with hypercalciuria, kidney stones, high blood pressure, osteoporosis and sensitivity to diuretic-induced hyperkalaemia.

17. The method of claim 15 wherein said protein sample is analyzed for the presence of a human TSC protein that is altered for one or more amino acids when compared to a wild-type human TSC protein.

18. The method of claims 17, wherein said protein sample is analyzed for the presence of a human TSC protein whose amino acid sequence is altered for one or more amino acids from wild-type TSC sequences.

19. The method of claim 18 wherein said protein sample is analyzed for the presence of a human TSC protein with an amino acid sequence selected from the group consisting of altered TSC sequences.

20. The method of claim 15 wherein said protein sample is analyzed for the presence of a human ROMK protein that is altered for one or more amino acids when compared to a wild-type human ROMK protein.

21. The method of claims 20, wherein said protein sample is analyzed for the presence of a human ROMK protein whose amino acid sequence is altered for one or more amino acids from wild-type ROMK sequences.

22. The method of claim 21 wherein said nucleic acid sample is analyzed for the presence of a nucleic acid molecule encoding a human ROMK protein with an amino acid sequence selected from the group consisting of altered ROMK sequences.

23. The method of claim 15 wherein said nucleic acid sample is analyzed for the presence of a nucleic acid molecule encoding a human NKCC2 protein that is altered for one or more amino acids when compared to a wild-type human NKCC2 protein.

24. The method of claims 23, wherein said protein sample is analyzed for the presence of a human NKCC2 protein whose amino acid sequence is altered for one or more amino acids from a wild-type NKCC2 sequence.

25. The method of claim 24 wherein said protein sample is analyzed for the presence of a human NKCC2 protein with an amino acid sequence selected from the group consisting of altered NKCC2 sequences.

26. The method of claim 17 wherein said altered TSC protein is analyzed using a method selected from the group consisting of gel-electrophoretic mobility, isoelectric point and binding to an alteration specific antibody.

27. The method of claim 20 wherein said altered ROMK protein is analyzed using a method selected from the group consisting of gel-electrophoretic mobility, isoelectric point and binding to an alteration specific antibody.

28. The method of claim 23 wherein said altered NKCC2 protein is analyzed using a method selected from the group consisting of gel-electrophoretic mobility, isoelectric point and binding to an alteration specific antibody.

29. An antibody that binds to an altered human TSC protein but not to a wild-type human TSC protein.

30. The antibody of claim 29, wherein said antibody does not bind a human TSC protein whose amino acid sequence is selected from the group consisting of wild-type TSC sequences.

31. The antibody of claim 30, wherein said antibody binds to an altered human TSC protein with an amino acid sequence selected from the group consisting of altered TSC sequences.

32. An antibody that binds to an altered human ROMK protein but not to a wild-type human ROMK protein.

33. The antibody of claim 32, wherein said antibody does not bind a human ROMK protein whose amino acid sequence is selected from the group consisting of wild-type ROMK sequences.

34. The antibody of claim 33, wherein said antibody binds to an altered human ROMK protein with an amino acid sequence selected from the group consisting of altered ROMK sequences.

35. An antibody that binds to an altered human NKCC2 protein but not to a wild-type human NKCC2 protein.

36. The antibody of claim 35, wherein said antibody does not bind a human NKCC2 protein whose amino acid sequence is selected from the group consisting of wild-type NKCC2 sequences.

37. The antibody of claim 36, wherein said antibody binds to an altered human NKCC2 protein with an amino acid sequence selected from the group consisting of altered NKCC2 sequences.

38. An isolated nucleic acid molecule that encodes a human protein selected from the group consisting of wild-type TSC protein, altered TSC protein, wild-type NKCC2 protein, altered NKCC2 protein and altered ROMK protein.

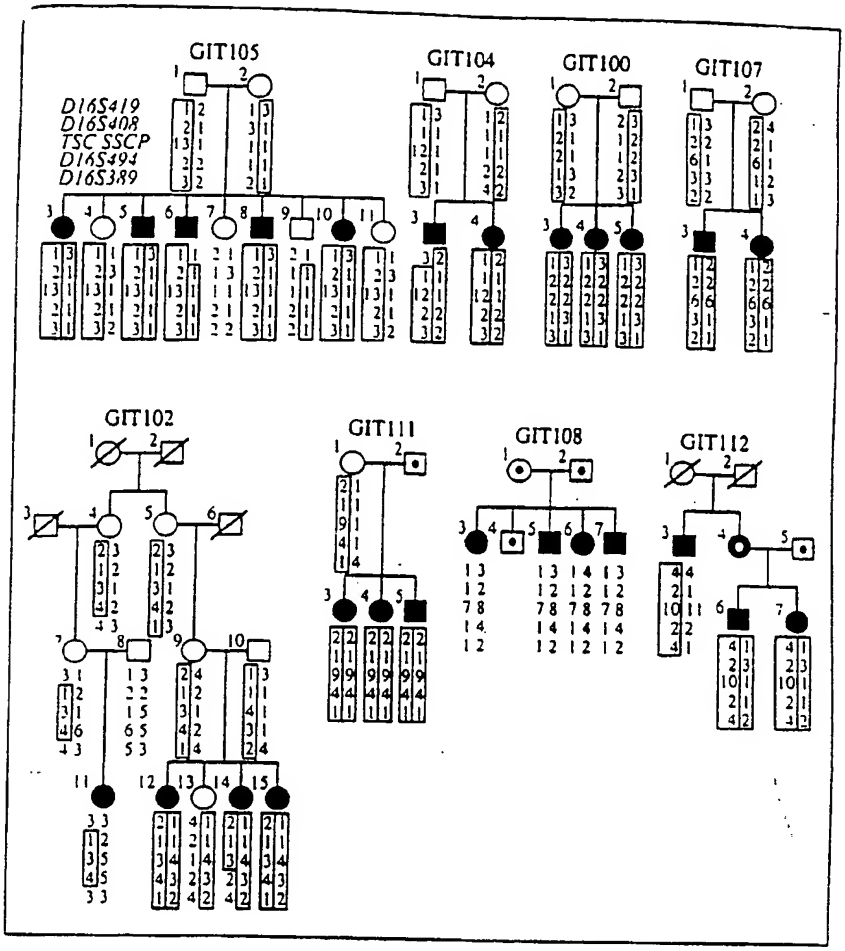
39. A vector containing a nucleic acid molecule according to claim 38.

40. A host cell transformed to contain a nucleic acid molecule according to claim 39.

41. A method for producing an ion transport protein comprising culturing the host of claim 40 under condition in which said nucleic acid molecule is expressed to produce a protein encoded thereby.

42. A method of identifying an agent the effects ion transport comprising the step of determining whether said agent binds to a protein produce by the method of claim 41.

43. An isolated protein with an amino acid sequence selected from the group consisting of human wild-type TSC protein, human altered TSC protein, human wild-type NKCC2 protein, human altered NKCC2 protein and human altered ROMK protein.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D16S408
TSC SSCP
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GIT107
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GIT108
GIT112

Figure 1

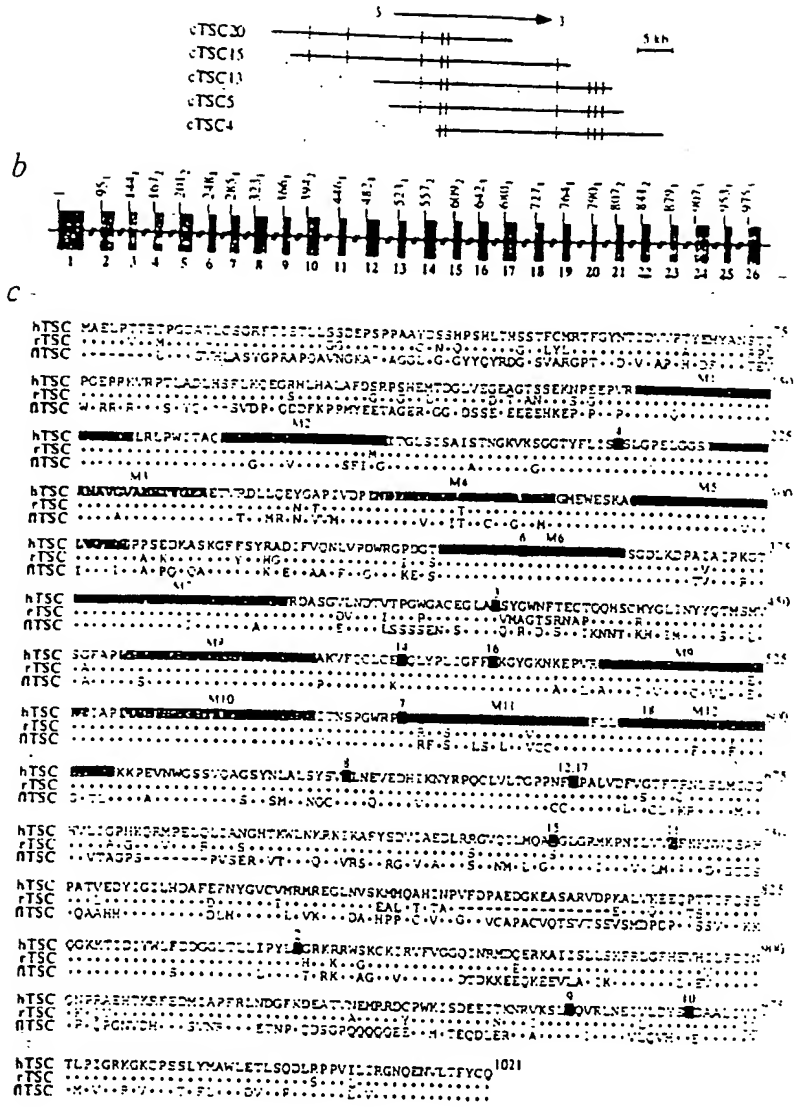


Figure 2

```
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 121 agcagccacc ccagccacct gacccacagc agcaccttct gcatgcgcac ctttggctac
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 481 ctgccctgga ttacggccca ggcaggcatc gtcctgacct ggatcatcat cctgctgtcg
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2101 tggctgaaca agaggaagat caaggccttc tactcggatg tcattgccga ggacctccgc
2161 agaggcgtcc agatcctcat gcaggccgca ggtctcggga gaatgaagcc caacattctg
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2941 gctgctctca tcgtcatcac tttgcccata gggaggaagg ggaagtgccc cagctcgctg
3001 tacatggcct ggctggagac cctgtcccag gacctcagac ctccagtcat cctgatccga
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3121 tccacagctc t
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FIGURE 2 (cont'd)

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 121 eagtsseknp eepvrfgwvk gvmircmlni wgvilylrlp witaqagivl twiiillsvt
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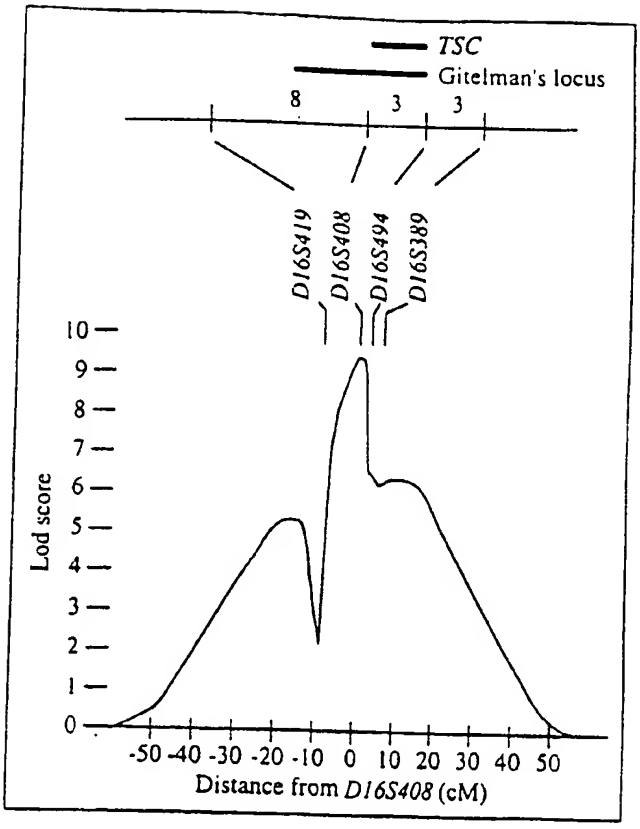
FIGURE 2 (cont'd)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Gitelman's locus
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3
3
D16S419
D16S408
D16S494
D16S389
Lod score
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9
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3
2
1
0
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Distance from D16S408 (cM)

Figure 3

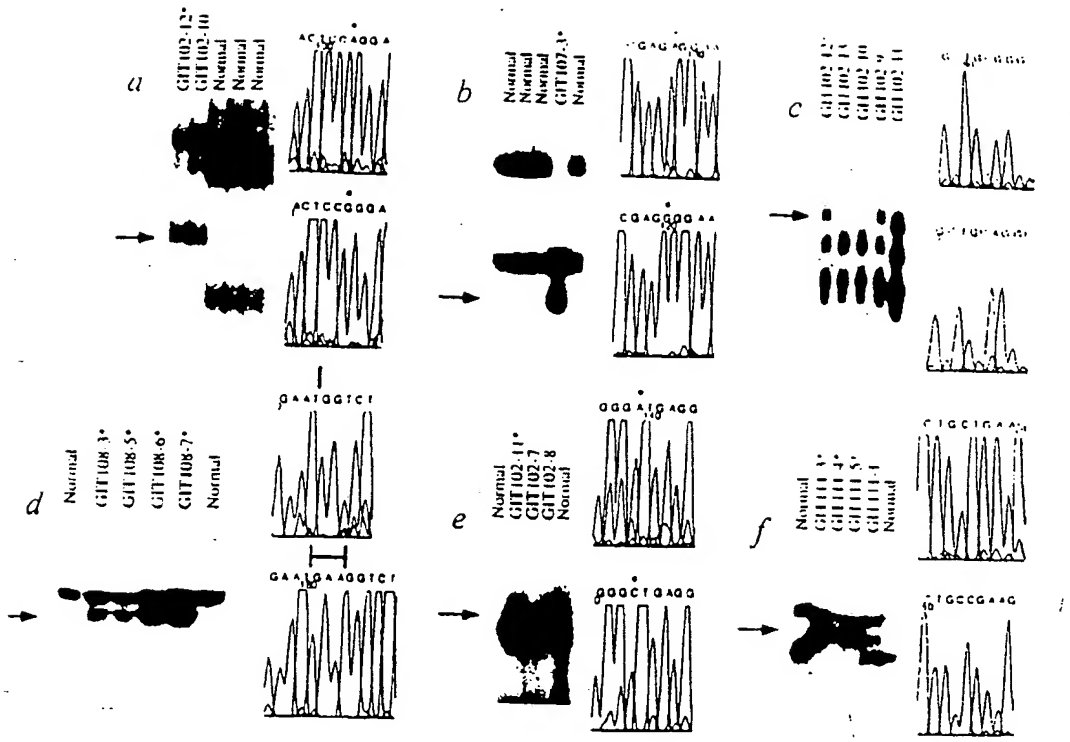


Figure 4

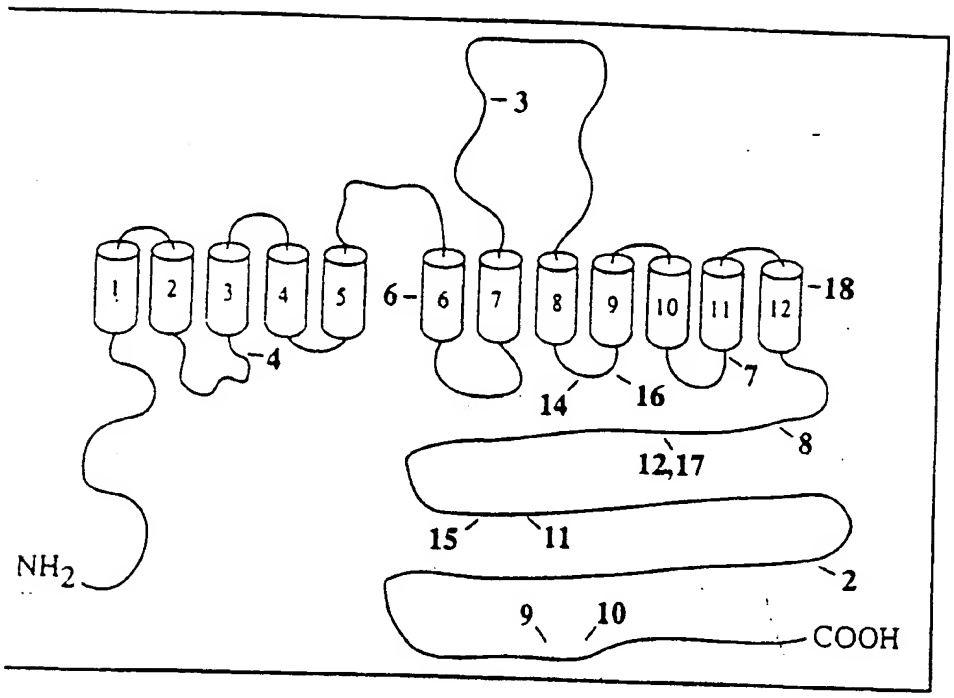


Figure 5

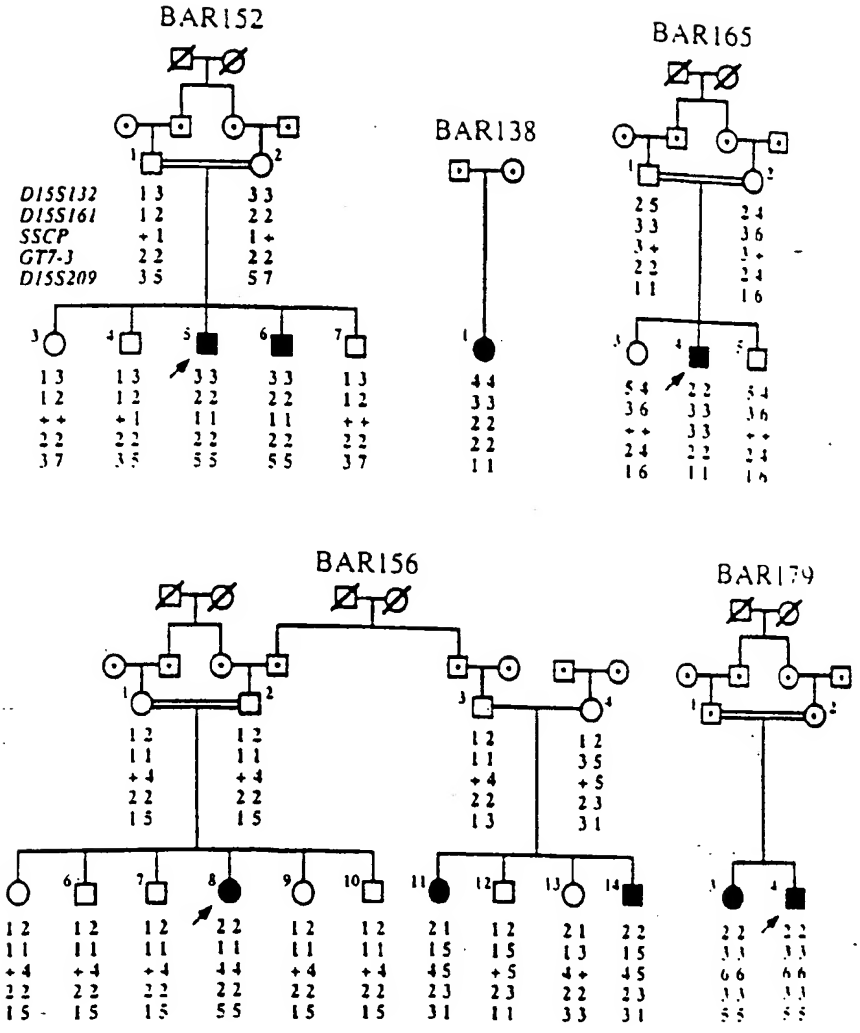
BAR152
D15S132
D15S161
SSCP
GT7-3
D15S209
BAR138
BAR165
BAR156
BAR179

Figure 6

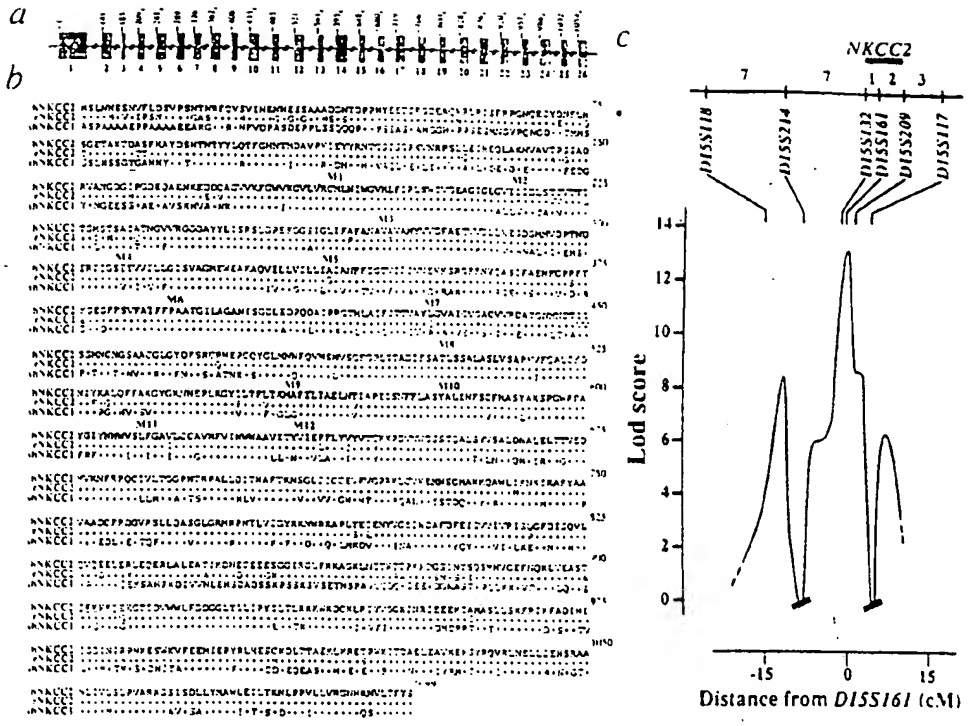
a
b
c
NKCC2
D15S118
D15S214
D15S132
D15S161
D15S209
D15S117
Lod score
Distance from D15S161 (cM)

Figure 7

```
   1 aaaaaatcaa ttttggaaga tgtcactgaa caactcttcc aatgtatttc tggattcagt
  61 gcccagtaat accaatcgct ttcaagttag tgtcataaat gagaaccatg agagcagtgc
 121 agctgcagat gacaatactg acccaccaca ttatgaagaa acctcttttg gggatgaagc
 181 tcagaaaaga ctcagaatca gctttaggcc tgggaatcag gagtgctatg acaatttcct
 241 ccacagtgga gaaactgcta aaacagatgc cagttttcac gcttatgatt ctcacacaaa
 301 cacatactat ctacaaactt ttggccacaa caccatggat gccgttccca agatagagta
 361 ctatcgtaac accggcagca tcagtgggcc caaggtcaac cgacccagcc tgcttgagat
 421 tcacgagcaa ctcgcaaaga atgtggcagt caccccaagt tcagctgaca gagttgctaa
 481 cggtgatggg atacctggag atgaacaagc tgaaaataag gaagatgatc aagctggtgt
 541 tgtgaagttt ggatgggtga aaggtgtgct ggtaagatgc atgctgaaca tctggggagt
 601 catgctcttc attcgccttt cctggattgt tggagaagct ggaattggtc ttggagttat
 661 catcattggc ctatccacca tagtaacgac aatcacaggt atgtccacgt ctgctattgc
 721 cacgaacgga gttgttagag gaggtggggc ctactatctt atttccagaa gtttagggcc
 781 cgagttcggt gggtcaatag gcctgatctt tgcttttgct aatgcagtgg ctgttgctat
 841 gtatgtggtg ggattcgctg aaactgtagt agatctactt aaggagagtg attcgatgat
 901 ggtggatcca accaatgaca tccggattat aggctccatc acagtggtga ttctttctagg
 961 aatttcagta gctggaatgg aatgggaggc aaaggcccaa gtcattcttc tggtcattct
1021 tctaattgct attgcaaact tcttcattgg aactgtcatt ccatccaaca atgagaaaaa
1081 gtccagaggt ttctttaatt accaagcatc aatatttgca gaaaactttg ggccacgctt
1141 cacaaagggt gaaggcttct tctctgtctt tgccattttt ttcccagcag ctactgggat
1201 tcttgctggt gccaatatct caggagattt ggaggatccc caagatgcca tccccagagg
1261 aaccatgctg gccattttca tcaccactgt tgcctactta ggggttgcaa tttgtgtagg
1321 ggcctgtgtg gtccgagatg ccaccgggaa catgaatgac accatcattt ctgggatgaa
1381 ctgcaatggt tcagcagcat gtgggttggg ctatgacttc tcaagatgtc gacatgaacc
1441 atgtcagtac gggctgatga acaatttcca ggtcatgagc atggtatcag ggttcggccc
1501 cctcatcact gcgggaatct tttctgcaac actctcctcc gccctggcct cccttgtcag
1561 cgcacccaaa gtgttccagg ctctgtgcaa ggacaacatc tacaaagccc tgcagttttt
1621 tgcaaaggga tatgggaaaa acaatgaacc cctgagagga tatattctca cttttcttat
1681 agccatggca tttattctta ttgcggaact gaacaccatt gctcccatca tctccaactt
1741 tttcctggcc tcatatgcac ttattaattt ctcctgcttc catgcctctt atgccaaatc
1801 tccaggatgg agacctgcgt atggaattta caacatgtgg gtatctcttt ttggagctgt
1861 tttgtgctgt gcagtcatgt ttgtcatcaa ctggtgggca gctgtcatca cctatgtcat
1921 tgaattcttc ctttacgtct atgtgacttg taagaagcca gatgtgaact ggggctcctc
1981 cacacaggct ctttcctacg tgagtgcttt agacaatgct ctggaattaa ccacagtgga
2041 agaccacgta aaaaacttca ggccccagtg cattgtctta acagggggac ccatgacaag
2101 acctgctctc ctggacataa ctcacgcctt taccaagaac agtggccttt gcatctgctg
2161 tgaagtcttt gtgggaccgc gcaaactgtg tgttaaggag atgaacagtg gcatggcgaa
2221 aaaacaggcc tggcttataa agaacaaaat caaggctttt tatgctgcag tggcggcaga
2281 ctgtttcagg gatggtgtcc gaagtcttct tcaggcctca ggcttaggaa gaatgaaacc
2341 aaacactctg gtgattggat ataagaaaaa ctggaggaaa gctcccttga cagagattga
2401 gaactacgtg ggaatcatac atgatgcatt tgattttgag attggcgtgg ttatagtcag
2461 aatcagccaa ggatttgaca tctctcaggt tcttcaggtg caagaggaat tagagagatt
2521 agaacaggag agactagcat tggaagcgac tatcaaagat aatgagtgtg aagaggaaag
2581 tggaggcatc cgaggcttgt ttaaaaaagc tggcaagttg aacattacta agacaacgcc
2641 taaaaaagat ggcagcatta acacaagcca gtcgatgcat gtgggagagt tcaaccagaa
2701 actggtggaa gccagcactc aatttaaaaa gaaacaagaa aaaggcacaa ttgatgtttg
2761 gtggttgttt gatgatggag ggttaacact tcttatcccc tatatcttaa ctctcagaaa
2821 aaaatggaaa gactgtaaat taagaatcta tgtgggaggg aagatcaacc gcattgaaga
2881 agaaaaaatt gcaatggctt cccttctgag caaatttagg ataaaatttg cagacatcca
2941 tatcatcggt gacatcaaca ttaggccaaa caaagagagc tggaaagtct ttgaagagat
3001 gattgaacca tatcgtctcc atgaaagctg caaagattta acaactgctg agaaattaaa
3061 aagagaaact ccgtggaaaa ttacagatgc agaactggaa gcagtcaagg aaaagagtta
3121 ccgccaagtt cgactgaatg aactcttaca ggagcactcc agagctgcta atctcattgt
3181 cctgagcctt cccgtggcaa gaaagggatc catatcggat ttgttgtata tggcttggtt
3241 ggaaatcctc acaaagaacc tcccacctgt cttactagtt agaggaaatc acaaaaatgt
3301 cttgacattt tactcttaaa acatgaaaga ttggaataca ttttaactta atgtaatgca
3361 ta
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FIGURE 7 (Cont'd)

```
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/product="bumetanide-sensitive Na-K-2Cl cotransporter"
/db_xref="PID:g1373425"
   1 mslnnssnvf ldsvpsntnr fqvsvinenh essaaaddnt dpphyeetsf gdeaqkrlri
  61 sfrpgnqecy dnflhsgeta ktdasfhayd shtntyylqt fghntmdavp kieyyrntgs
 121 isgpkvnrps lleiheqlak nvavtpssad rvangdgipg deqaenkedd qagvvkfgwv
 181 kgvlvrcmln iwgvmlfirl swivgeagig lgviiiglst ivttitgmst saiatngvvr
 241 gggayylisr slgpefggsi glifafanav avamyvvgfa etvvdllkes dsmmvdptnd
 301 iriigsitvv illgisvagm eweakaqvil lvilliaian ffigtvipsn nekksrgffn
 361 yqasifaenf gprftkgegf fsvfaiffpa atgilagani sgdledpqda iprgtmlaif
 421 ittvaylgva icvgacvvrd atgnmndtii sgmncngsaa cglgydfsrc rhepcqyglm
 481 nnfqvmsmvs gfgplitagi fsatlssala slvsapkvfq alckdniyka lqffakgygk
 541 nneplrgyil tfliamafil iaelntiapi isnfflasya linfscfhas yakspgwrpa
 601 ygiynmwvsl fgavlccavm fvinwwaavi tyviefflyv yvtckkpdvn wgsstqalsy
 661 vsaldnalel ttvedhvknf rpqcivltgg pmtrpalldi thaftknsgl ciccevfvgp
 721 rklcvkemns gmakkqawli knkikafyaa vaadcfrdgv rsllqasglg rmkpntlvig
 781 ykknwrkapl teienyvgii hdafdfeigv vivrisqgfd isqvlqvqee lerleqerla
 841 leatikdnec eeesggirgl fkkagklnit kttpkkdgsi ntsqsmhvge fnqklveast
 901 qfkkkqekgt idvwwlfddg gltllipyil tlrkkwkdck lriyvggkin rieeekiama
 961 sllskfrikf adihiigdin irpnkeswkv feemiepyrl hesckdltta eklkretpwk
1021 itdaeleavk eksyrqvrln ellqehsraa nlivlslpva rkgsisdlly mawleiltkn
1081 lppvllvrgn hknvltfys
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FIGURE 7 (Cont'd)

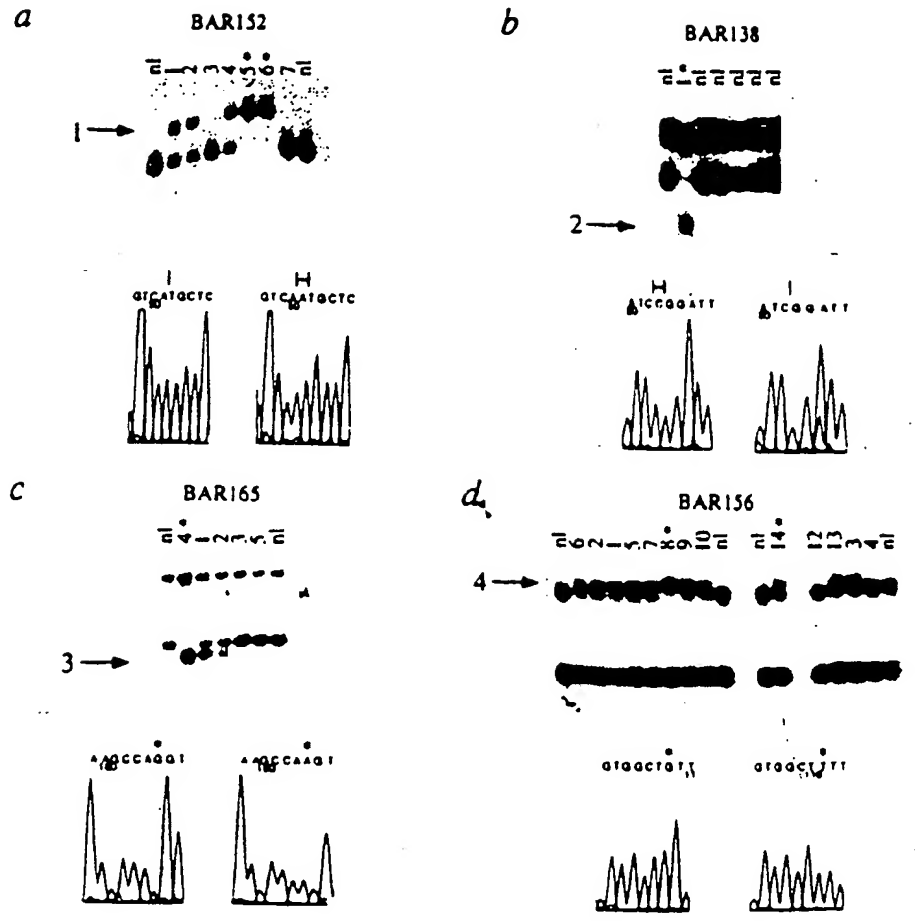


Figure 8

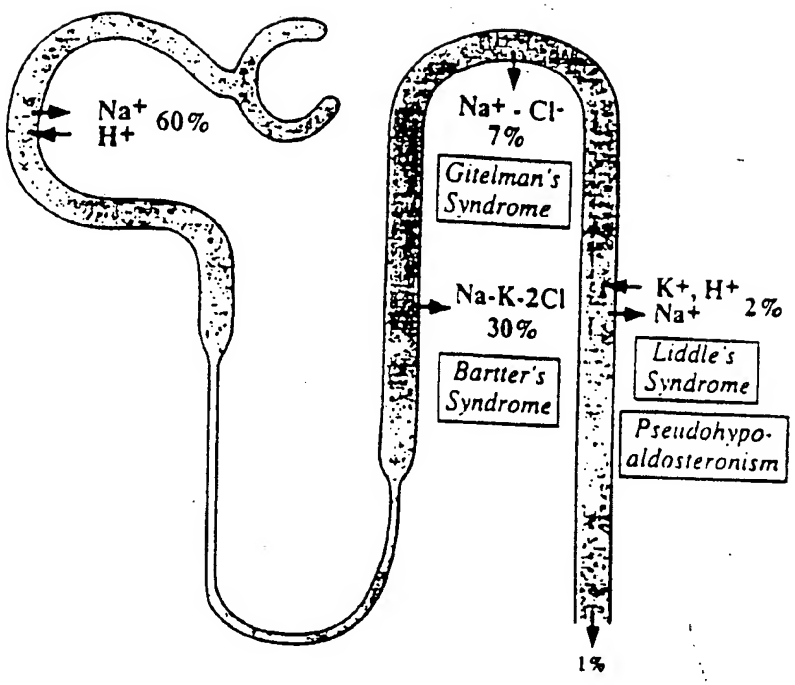
Na+
H+ 60%
Na+ - Cl-
7%
Gitelman's Syndrome
Na-K-2Cl
30%
Bartter's Syndrome
K+, H+
Na+ 2%
Liddle's Syndrome
Pseudohypo-aldosteronism
1%

Figure 9

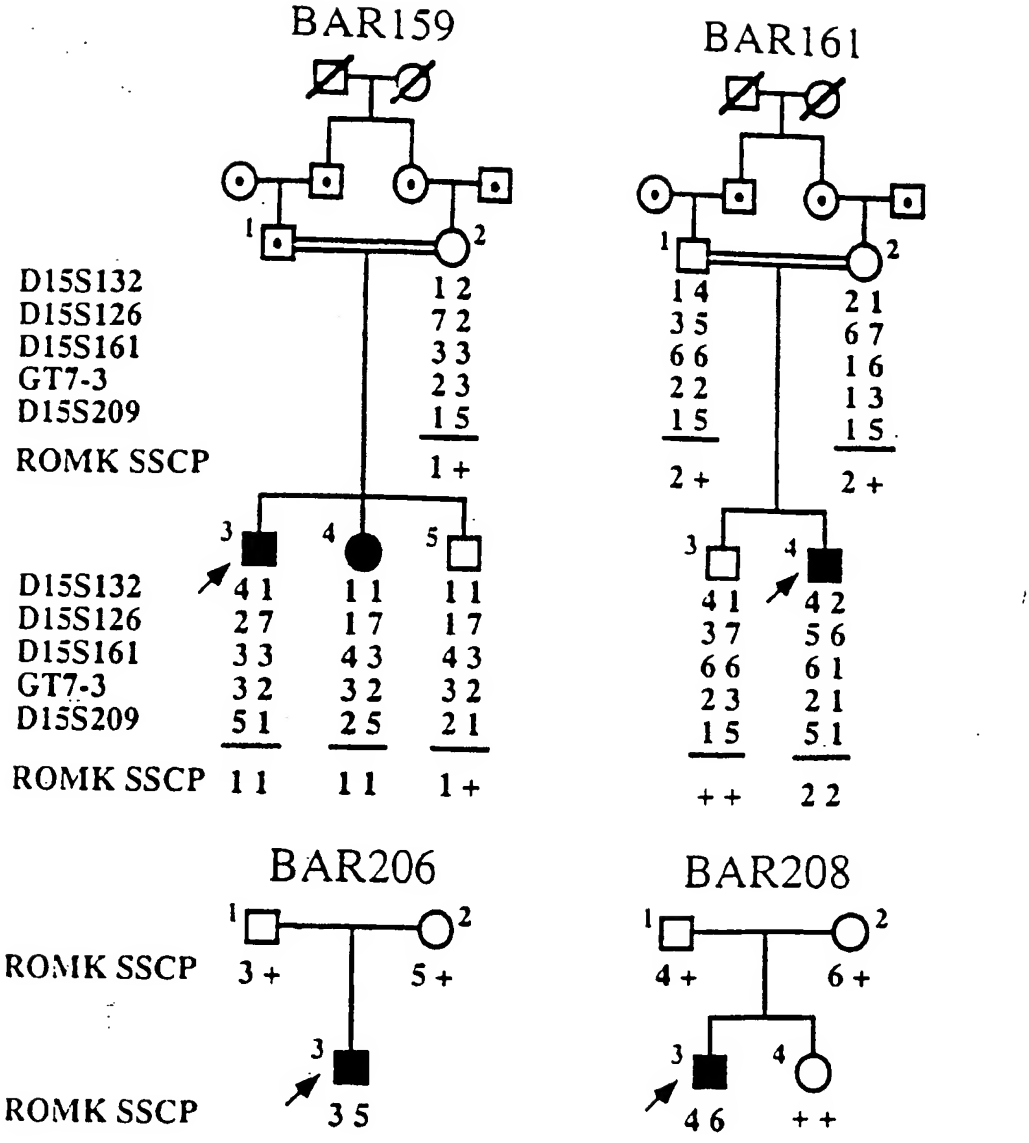
BAR159
D15S132
D15S126
D15S161
GT7-3
D15S209
ROMK SSCP
1 2
7 2
3 3
2 3
1 5
1+
D15S132
D15S126
D15S161
GT7-3
D15S209
ROMK SSCP
4 1
2 7
3 3
3 2
5 1
1 1
1 1
1 7
4 3
3 2
2 5
1 1
1 1
1 7
4 3
3 2
2 1
1+
BAR161
1 4
3 5
6 6
2 2
1 5
2+
2 1
6 7
1 6
1 3
1 5
2+
4 1
3 7
6 6
2 3
1 5
++
4 2
5 6
6 1
2 1
5 1
2 2
BAR206
ROMK SSCP
3+
5+
ROMK SSCP
3 5
BAR208
4+
6+
4 6
++

Figure 10

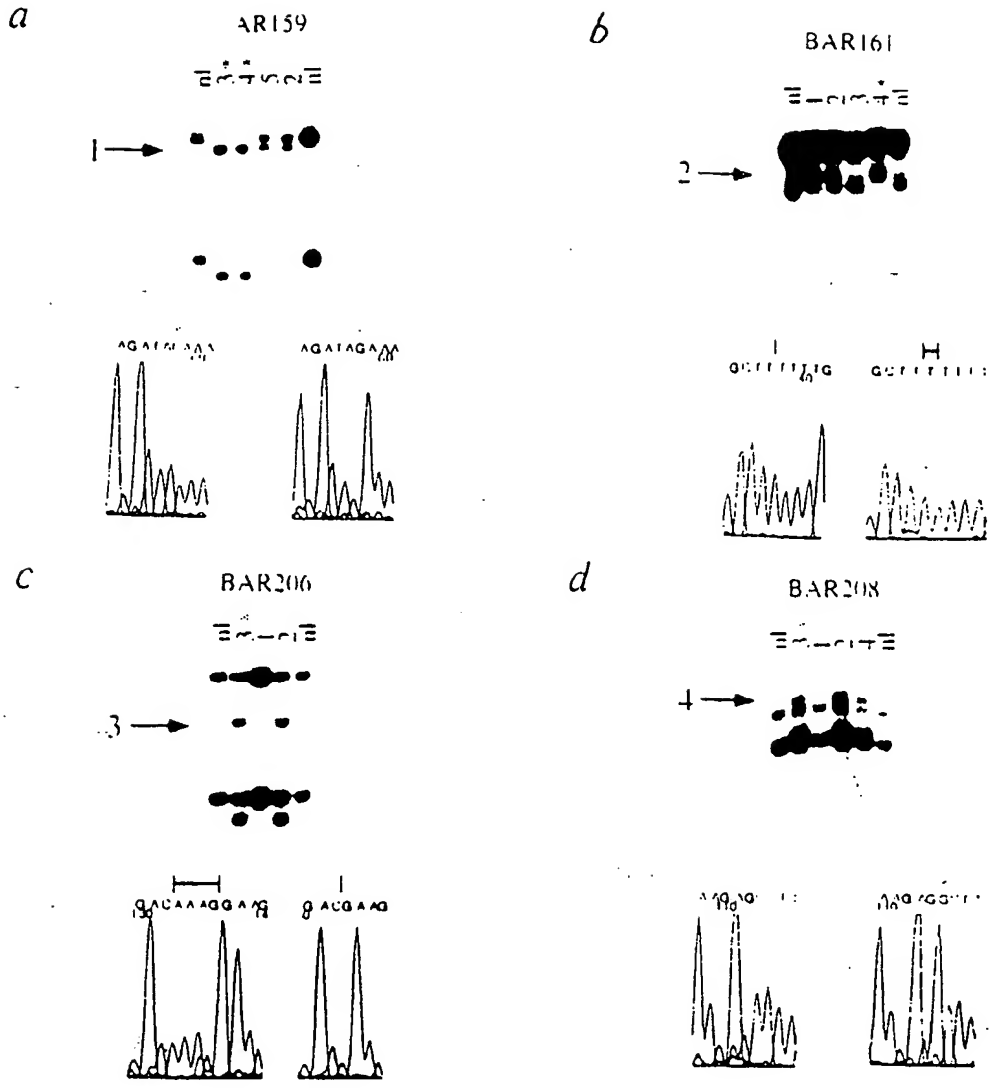


Figure 11

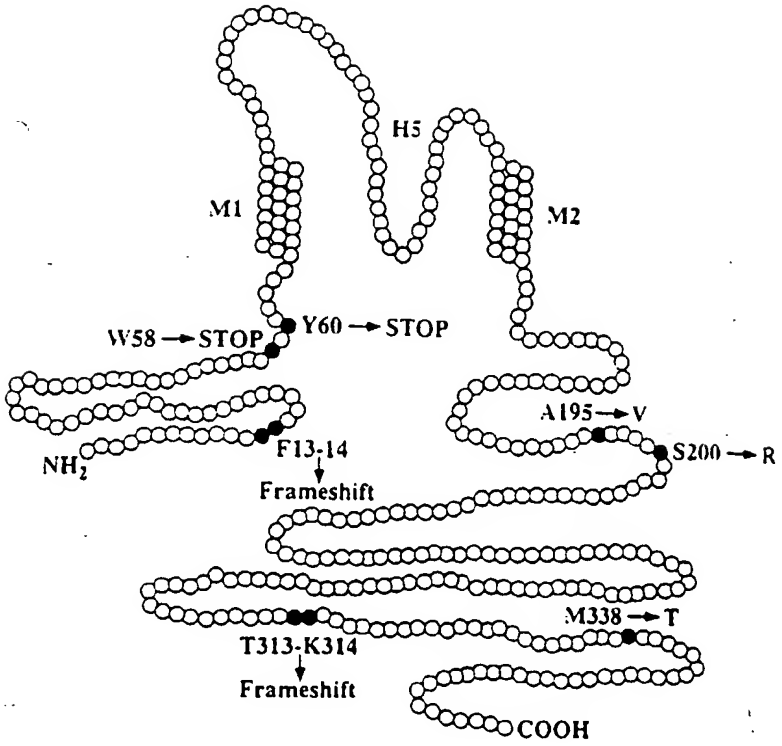
H5
M1
M2
W58 → STOP
Y60 → STOP
A195 → V
S200 → R
NH2
F13-14
Frameshift
M338 → T
T313-K314
Frameshift
COOH

Figure 12

# INTERNATIONAL SEARCH REPORT

International application No.
PCT/US97/23553

A. CLASSIFICATION OF SUBJECT MATTER

IPC(6) :C07H 21/04; C12Q 1/68; C07K 14/435, 16/00; G01N 33/53

US CL :Please See Extra Sheet.

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

U.S. : 435/4, 6, 7.1, 91.2, 252.3, 320.1; 536/23.5, 24.31, 24.33; 530/350, 388.2

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

NONE

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

Please See Extra Sheet.

C. DOCUMENTS CONSIDERED TO BE RELEVANT

| Category* | Citation of document, with indication, where appropriate, of the relevant passages | Relevant to claim No. |
|---|---|---|
| Y | MASTROIANNI et al. Novel molecular variants of the Na-Cl cotransporter gene are responsible for Gitelman Syndrome. American Journal of Human Genetics. November 1996, Vol. 59, pages 1019-1026, especially page 1020 and figure 2. | 1-5, 12, 15-19, 26, 29-31, 38-43 |
| Y | YANO et al. Alternative splicing of human inwardly rectifying $K^+$ channel ROMK1 mRNA. Molecular Pharmacology. May 1994, Vol. 45, pages 854-860, especially figure 1 and pages 858-859. | 32-34, 38-41, 43 |

[X] Further documents are listed in the continuation of Box C. [ ] See patent family annex.

| | | | |
|---|---|---|---|
| * | Special categories of cited documents: | "T" | later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention |
| "A" | document defining the general state of the art which is not considered to be of particular relevance | "X" | document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone |
| "E" | earlier document published on or after the international filing date | "Y" | document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art |
| "L" | document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified) | "&" | document member of the same patent family |
| "O" | document referring to an oral disclosure, use, exhibition or other means | | |
| "P" | document published prior to the international filing date but later than the priority date claimed | | |

| | |
|---|---|
| Date of the actual completion of the international search<br>23 MARCH 1998 | Date of mailing of the international search report<br>19 MAY 1998 |
| Name and mailing address of the ISA/US<br>Commissioner of Patents and Trademarks<br>Box PCT<br>Washington, D.C. 20231<br>Facsimile No. (703) 305-3230 | Authorized officer<br>CARLA MYERS<br>Telephone No. (703) 308-0196 |

Form PCT/ISA/210 (second sheet)(July 1992)*

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US97/23553

C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

| Category* | Citation of document, with indication, where appropriate, of the relevant passages | Relevant to claim No. |
|---|---|---|
| Y | IGARASHI et al. Cloning, embryonic expression, and alternative splicing of a murine kidney-specific Na-K-Cl cotransporter. American Journal of Physiology. September 1995, Vol. 269, pages F405-F418, especially pages F406-F407. | 35-41, 43 |
| Y | SIMON et al. Gitelman's variant of Bartter's syndrome, inherited hypokalaemic alkalosis, is caused by mutations in the thiazide-sensitive Na-Cl cotransporter. Nature Genetics. January 1996, Vol. 12, pages 24-30, especially page 26. | 1-5, 12, 15-19, 26, 29-31, 38-43 |
| Y | SIMON et al. Bartter's syndrome, hypokalaemic alkalosis with hypercalciuria, is caused by mutations in the Na-K-2Cl cotransporter *NKCC2*. Nature Genetics. June 1996, Vol. 13, pages 183-188, especially pages 187-188 and figure 2. | 1, 2, 9-11, 14-16, 23-25, 28, 35-43 |

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US97/23553

A. CLASSIFICATION OF SUBJECT MATTER:
US CL :

435/4, 6, 7.1, 91.2, 252.3, 320.1; 536/23.5, 24.31, 24.33; 530/350, 388.2

B. FIELDS SEARCHED
Electronic data bases consulted (Name of data base and where practicable terms used):

APS; DIALOG: Medline, Biosis, Derwent Patents, CA, EMBASE
search terms: TSC, NCCT, SLC12A3, NKCC2, NKCC, Na-K-2Cl, ROMK, cotransporter, nucleic acid, DNA, probe, gene, protein, antibody, mutation, polymorphism, Gitelman's Syndrome, GS, Bartters Syndrome, hypokalaemic alkalosis, high blood pressure, hypercalciuria, hyperkalaemia.